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Sodium Chloride Depletion and Renal Concentrating Ability during Chronic Administration of Thiazide (Centyl ®) and Furosamide (Lasix ®) to the Normal Rat

By

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(Received September 9 1968)

An important problem in the evaluation of the mode of action of natriuretic agents in the long term treatment of non-oedematous subjects (hypertensive patients), is the duration of the natriuretic effect caused by the drugs. Thus for example do these agents maintain a constant sodium chloride depletion in a patient receiving a constant amount of sodium in the diet, or is the sodium deficit gradually restored in spite of continued treatment? This problem is also of interest for the evaluation of the renal mode of action of the drug, and for the evaluation of the compensatory mechanisms limiting sodium depletion. Very little information seems to be available on the problem outlined above. To the authors' knowledge there are no reports on the sodium depleting effect of *chronic* natriuretic treatment in normal subjects or animals.

In a study of patients with essential hypertension receiving chronic thiazide treatment it was not possible to demonstrate any decrease in the total exchangeable sodium nor was it possible to demonstrate a decrease in the extracellular fluid during the chronic phase of a thiazide treatment (LAUWERS & CONWAY 1960 GIFFORD *et al* 1961). Thus the patients would seem make good the initial loss of sodium chloride during a long term treatment. In contrast to this observation salt depletion was observed in an investigation on rats with diabetes insipidus: this depletion was maintained quantitatively throughout a long period of thiazide treatment (SKADHAUGE 1966). The initial salt loss caused by the drug was not compensated for until after the discontinuation of the thiazide treatment. Diabetes insipidus rats may however not respond as normal rats. Therefore, in the present study chronic thiazide and furosamide treatment was given to normal rats, and the sodium chloride balance followed.

There is a discrepancy in the literature about the possible effects of acute thiazide administration on renal concentrating ability. In acute thiazide experiments on hydropenic dogs in mannitol or isotonic saline diuresis, no appreciable changes in $T^{\text{C}}_{\text{H}_2\text{O}}$ have been found (EARLEY *et al.* 1961, SELDIN *et al.* 1966). $T^{\text{C}}_{\text{H}_2\text{O}}$ is defined as

$$\frac{\text{Urine Osmolality} \times \text{Urine Volume}}{\text{Plasma Osmolality} \times \text{Urine Volume}}$$

This value is often used as a measure of the renal concentrating ability. Similar investigations on hydropenic rats have shown that acute thiazide medication results in a significant decrease in $T^{\text{C}}_{\text{H}_2\text{O}}$ (KOSTNER 1965). To assess the effect of chronic thiazide treatment on the concentrating mechanism of the kidney, we have measured the osmotic urine to plasma ratio during chronic administration. In addition, we have performed separate experiments on dehydrated animals, in order to find out whether the chronic thiazide treatment impairs the maximal concentrating ability as determined by urine osmolality.

Material and Methods

Metabolic experiments. White male rats of The Danish State Serum Institute stock weighing 200–300 grams were used. The rats were kept in individual metabolic cages. The urine was collected for 24 hr periods under kerosene with the addition of thymol. Throughout the experiments the rats drank demineralised water *ad libitum*. The water bottles were fitted outside the cages in order to prevent dilation of the urine. Sixteen g of food were offered daily as powder thoroughly mixed with 16.0 ml of 50 mM NaCl solution, which made the food paste-like. The water content of the powdered food was 15%. The food was mixed in a glass beaker which was placed in a vertical cylinder attached to the outside of the cage. Loss of more than a few mg of food was thus prevented. The food that was left over was weighed after 24 hrs, drying at 120°. The rats normally ingested 13–16 g food daily. The estimation of the daily food and electrolyte ingestion is accurate within ±5%. Faecal contamination of the urine was avoided by placing a faeces collecting grid and a disposable plastic funnel under the cages. The faeces collecting grids and the plastic funnels were replaced after the faeces had been removed and the grids and the plastic subsequently rinsed with a weighed amount of demineralised water (approximately 100 ml). The salt loss in the cage was negligible. The faeces collecting grids were cleaned in tap water with a detergent and rinsed in demineralised water. Aliquots of the mixed 24 hr urine and of the rinsing water were stored for a few days at 4° until analysis. Food was offered and the cages cleaned daily at 3 p.m. Most experiments were performed as follows. After a training period of 3–4 days in the metabolic cages, a 4–6 day control period was followed by a 6 day period of treatment. This was followed by a 4–6 day control period. This allowed every treatment period to be compared with an average of an 8–12 day control period. The maintenance of the rats and all the weighings were performed by one of us (K.S.).

Dehydration Experiments. Two groups of male white rats weighing 220–250 g were used. One group received the standard salt load containing a supramaximal dose of

bendroflumethiazide, the other group, the control group, received the same salt load without thiazide. After an initial period of 72 hours, during which the animals were offered the above mentioned food and demineralised water to drink ad libitum, there followed a 46 hour dehydration period. In order to ensure a supramaximal treatment of the thiazide group throughout the dehydration period, the two groups of rats, after 22 hours, are offered the same food as they each had received in the initial period. Urine was collected in the following periods. The first period was during 22-28½ hours dehydration, the second one was during 28½-41 hours dehydration and the third period during 41-46 hours of dehydration. The rats were weighed initially and also after 20, 27, 40 and 46 hours. Urine osmolalities were measured as soon as the last urine collection period had been completed. After this experiment the rats were not used for any further experiments for 5 days.

Blood sampling. Blood loss and the disturbance involved in venepuncture might affect the sodium balance and the renal concentrating capacity. In separate experiments on 18 rats blood was therefore drawn under light ether anaesthesia from the exposed external jugular vein. Blood was collected in heparinized glass tubes. Plasma sodium, potassium, chloride and osmolality were measured. The latter was used to calculate the urine to plasma ratio and $T\bar{C}r$. Venepuncture was performed only once a week and after the rats had received the diet for three days. These rats were not used for balance studies.

Feed, Drugs. Throughout the experiment a 100 kg batch (Altromin ®) rat powder was used. According to the manufacturer the electrolyte content of the food was sodium 52, potassium 112, chloride 80 mEq/kg. The composition of the food had been controlled previously (SKADHAUG 1966). Up to 5% deviation was observed between the values given by the manufacturer and the values found by metabolic control experiments with no added electrolytes (SKADHAUG 1966). The electrolyte solution added to the content of the food, contained sodium 100 µEq/g food, and chloride 130 µEq/g food. The strong electrolytes account for slightly less than one third of the total urine osmolality and area for two thirds. In order to ensure good mixing of the drugs in the food, appropriate amounts of the drugs used in the study were first mixed with finely ground food powder after which this mixture was thoroughly mixed with the food powder in an electric blender in a ratio of 10% (W/W). Bendroflumethiazide (Cenyl ® Leo Pharmaceutical Comp.) was given in a dose of 0.1 mg/g food. Furosemide (Lasix ® Hoechst) was given a dose of 4.9 mg/g food. These doses are larger than those required to produce a maximal natriuretic effect (KROEMER 1960, THURMANN 1964).

Analyses. Urine and plasma osmolality was measured by freezing point depression on Knauer's semimicro-osmometer on 0.150 ml samples (urine) and on an Advanced Instruments osmometer on 0.250 ml samples (plasma) using the same NaCl standards. Chloride was determined by mercurimetric titration (BAUM 1949). Sodium and potassium were analysed on the Eppendorf flame-photometer. The standards for the urine analyses, like the urine samples, contained equal amounts of sodium and potassium. Most analyses were performed in duplicate, but the samples from the first day of treatment and the first days after discontinuation of treatment are, however, analysed in triplicate.

Calculations. In the rat changes in daily salt intake are compensated for by urinary excretion within 24 hrs. (SKADHAUG 1966). A new steady state is reached within this time limit. For this reason the daily electrolyte excretion in the metabolic experiments was expressed in µEq/g food / 24 hrs. This corrects for errors in estimation of electrolyte output due to minor changes in food intake. Statistical calculations were made according to KEMP & NIELSEN (1961).

Control Experiments. By using artificial urines (NaCl + KCl + Urea solutions) it was shown that the method of urine collection resulted in a volume loss of 30 % for the standard

salt load with thiazide or furosamide medication. An osmolality increase of respectively 5 and 4% was measured. No changes in volume and osmolality were observed after collection of the samples into the bottles. The addition of thymol prevented bacterial decomposition, while in itself causing an osmolality increase of 4-5%. Thus the errors in osmolality induced by the urine collection procedure were small and constant. The rinsing procedure ensured an almost complete recovery of the voided amount of electrolytes.

Results

A Plasma values

As shown in table 1 the administration of thiazide as well as furosamide resulted in a significant decrease in plasma chloride and potassium concentrations, but no significant changes in plasma osmolalities were observed.

II Thiazide experiments.

1 The effects of thiazide on the excretion of sodium chloride and potassium in normal rats

After an initial 3 day control period 10 normal rats were given thiazide for 6 days, followed by a second control period of 6 days duration. The average daily body weight and urinary excretion of sodium chloride and potassium are shown in fig. 1. On the first day of administration, an increase in the excretion of sodium and chloride was observed, but no further difference from the control values was found during the remaining period of treatment. On the first day after discontinuation of the thiazide treatment a decrease in the excretion of these ions was observed. The

Table 1

Plasma osmolality and electrolyte concentrations.

Figures in brackets indicate number of rats. (Units mOsm and meq/l)

Parameters observed	Control group (17) Mean \pm S.D.	Thiazide administration (12) Mean \pm S.D.	Furosamide administration (6) Mean \pm S.D.
Na	141 \pm 4.5	139 \pm 5.65 N.S.	141 \pm 2.00 N.S.
K	2.86 \pm 0.45	2.40 \pm 0.36)	2.11 \pm 0.30)
Cl	101 \pm 4.70	94 \pm 3.21)	91 \pm 4.24)
Osm	294 \pm 5	292 \pm 7 N.S.	296 \pm 4 N.S.

Statistical significance from control :) 0.05 > P > 0.01 : 0.01 > P > 0.001
) P < 0.001 N.S. denotes not significant.

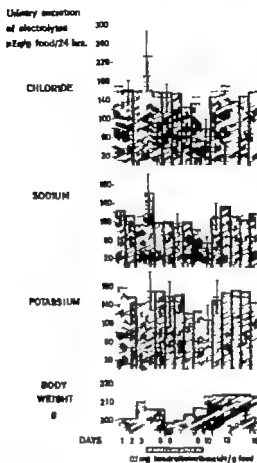


Fig. 1. Natriuretic effect of thiazide medication. Average values and standard deviations for 10 rats. The sodium chloride loss and retention occurred on the first day of drug administration and discontinuation respectively. A slight weight loss was seen.

average sodium and chloride excretions on the first day of treatment and on the first day of cessation of treatment were calculated and were all found to differ significantly from the average daily control excretion when tested by a *t* test ($P < 0.001$). Apart from these days no changes from the average daily control chloride excretion was seen. The sodium excretion was, however, slightly lower during the chronic period of administration. The values are shown in table 2.

For each rat the increase in sodium and chloride excretion on the first day of treatment was calculated, as was the decrease in excretion on the first day after discontinuation of treatment. The average values and the standard deviations of these four parameters were determined, and the average compared by means of *t*-tests. No significant differences from

Table 2

Urinary electrolyte excretion in the thiazide experiment
 The table includes 10 rats the units are $\mu\text{Eq/g food} / 24 \text{ hrs.}$

Average daily excretion	Control period Mean \pm S.D.	Chronic thiazide administration period Mean \pm S.D.	1 thiazide administration day Mean \pm S.D.	1 discontinuation day Mean \pm S.D.
N	113 \pm 28	97 \pm 21)	162 \pm 44)	55 \pm 13 *)
K	158 \pm 36	147 \pm 44 N.S.	170 \pm 41 N.S.	109 \pm 29)
Cl	157 \pm 25	146 \pm 29 N.S.	235 \pm 76 *)	81 \pm 19)

Statistical significance from control) $0.05 > P > 0.01$) $0.01 > P > 0.001$
 *) $P < 0.001$ N.S. denotes not significant.

zero were found. The standard deviations of the average values for the increase in sodium and chloride excretion on the first day of treatment were compared by means of an F test. No significant variations was observed, and a common variance was calculated. Similar calculations were made for the first day after discontinuation of thiazide administration and a common variance determined. The average value for the salt deficit found on the first day of treatment was $63.50 \mu\text{Eq/g dry food} / 24 \text{ hrs.}$ (S.D. 60.23) The average salt retention on the first day after discontinuation of treatment was $67.00 \mu\text{Eq/g food} / 24 \text{ hrs.}$ (S.D. 14.77) The average value for the salt deficit was compared with the average value for salt retention by means of a t-test. No significant difference was found ($P > 0.1$).

In order to determine whether the daily sodium excretion in the control periods was normally distributed, a χ^2 -test was performed. No significant difference was found indicating that the values were normally distributed ($0.95 < P < 0.1$). The sodium depletion amounted to 10% of exchangeable sodium and to approximately half of the average daily control sodium excretion. No change in food intake was observed. An average weight loss of 8 g occurred during thiazide treatment. Although no kaliuresis was obvious during the treatment period, a slightly but highly significant potassium retention was seen on the first discontinuation day (see table 2).

2. The effects of thiazide administration on the osmotic concentration of the urine in the normal rat

In the above mentioned experiment (1) 10 normal rats received the standard salt load and thiazide and the average daily values for $\text{TC}_{\text{H}_2\text{O}}$ and the osmotic urine to plasma ratio were calculated.

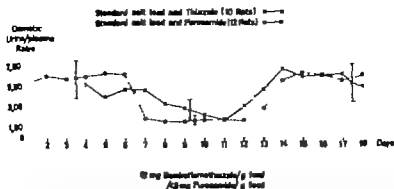


Fig. 2. The effect of thiazide and furosemide medication on the osmotic urine to plasma ratio. The rats received water *ad libitum*. Average values and standard deviations are shown. During the chronic administration periods significant decreases in the osmotic urine to plasma ratios were observed.

The average daily control T_{H_2O} was 45.31 (S.D. 12.01) ml/24 hrs. During the chronic medication period T_{H_2O} was 31.80 (S.D. 10.70) ml/24 hrs. The two values were compared by a *t*-test. A highly significant difference was found ($P < 0.001$), compatible with a decrease in the kidney's concentrating ability. The average daily osmotic U/P ratios and their standard deviations are shown in fig. 2. The average daily U/P ratio in the chronic thiazide treatment period was compared with the average daily control U/P ratio. The U/P ratio decreased during thiazide administration. A highly significant difference was found using a *t*-test ($P < 0.001$). The control periods involved a larger fractional volume loss of urine in the cages than in the chronic thiazide periods of treatment. In order to correct for this in the calculations of T_{H_2O} the daily control urine volumes were corrected to the same fractional volume loss as in the chronic thiazide medication period. No corrections were made in the osmotic U/P ratio calculations, since the errors resulting from the collection were small and constant.

In order to elucidate whether the observed decrease in the osmotic concentration of the urine during the chronic phase of the thiazide treatment was due to a primary increase in water intake or to a primary decrease in the kidney's concentrating ability a dehydration experiment was carried out.

3 Dehydration experiment

For each urine collection period the average osmotic urine to plasma (U/P) ratios and their standard deviations were calculated for both the

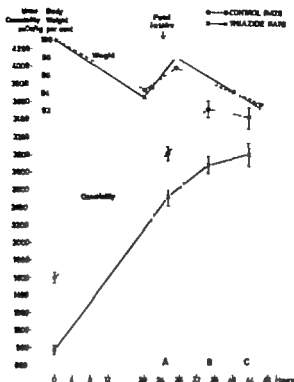


Fig. 3 The effect of thiazide on body weight and urine osmolality 12 rats received the diet alone and 12 rats were given the diet and chronic thiazide treatment. Average values and standard error of the mean are shown. At the same fractional weight loss as the control rats the thiazide treated rats showed a significant decrease in urine osmolality indicating a significant decrease in the renal concentrating capacity during the chronic drug administration.

thiazide treated group and the control group. The average rat weight in per cent of control body weight and mean U/P ratios and standard error of the mean of both rat groups in fig. 3 are related to the number of hours of dehydration. For each collection period the U/P ratios of the thiazide treated group and the control group were compared by *t* tests. In each test a significant difference in the U/P ratio was demonstrated (first $P < 0.001$ second $P < 0.001$ third $P < 0.01$). Since both groups of rats were dehydrated to the same fractional weight loss, the significant decrease in the U/P ratios of the thiazide treated group indicates that chronic administration of this drug causes a significant decrease in the concentrating ability of the rat kidney. The observed average maximum urinary osmotic pressure in the control group is in agreement with the value reported by SCHMIDT NIELSEN *et al* (1948).

Table 3

*Urinary electrolyte excretion in the long term thiazide experiment*The table includes 6 rats, the units are $\mu\text{Eq/g food} / 24 \text{ hrs.}$

Average daily excretion	Control period Mean \pm S.D.	Chronic thiazide administration period Mean \pm S.D.	1 thiazide administration day Mean \pm S.D.	1 discontinuation day Mean \pm S.D.
N	96 \pm 11	98 \pm 4 N.S.	123 \pm 11)	72 \pm 9 *)
K	135 \pm 18	151 \pm 19 N.S.	148 \pm 13 N.S.	119 \pm 5)
Cl	133 \pm 15	146 \pm 11 N.S.	178 \pm 21 6)	105 \pm 8)

Statistical significance from control) $0.05 > P > 0.01$ *) $0.01 > P > 0.001$) $P < 0.001$ N.S. denotes not significant.

4 The effect of a more prolonged thiazide administration period on the excretion of sodium chloride and potassium in the normal rat

6 normal rats were given a supramaximal thiazide medication for 21 days. This is approximately 3% of a rat's lifetime. The observations made in this experiment are in agreement with the results found in the 6-day experiment. The sodium chloride deficit established on the first day of treatment was, however slightly lower than in the 6-day experiment. The average daily weights and urinary excretions of sodium and chloride during the control period, the chronic thiazide medication period, the first day of treatment, and on the first day after discontinuation of the drug are shown in table 3. On the first day of treatment a significant increase in the excretion of sodium and chloride was demonstrated by comparing the average values of sodium and chloride excretion on the first day of treatment with the average daily control excretion values for these ions (sodium $P < 0.001$ chloride $P < 0.01$). Similarly the urinary excretion of these ions on the first day after discontinuation of the drug was significantly smaller than the average daily control excretion values (sodium $P < 0.001$ chloride $P < 0.001$). No change from average daily control values was seen beyond these days. (sodium $P > 0.1$ chloride $P > 0.1$). The average values for the increase in sodium and chloride excretion on the first day of treatment were compared. No significant difference was found using t-test ($P < 0.1$) indicating that the sodium loss on the first thiazide day was of the same magnitude as the chloride loss. In order to determine whether the difference between the increase in the average excretion of sodium and of chloride on the first day of drug

administration was significantly different from the decrease in the urinary excretion of these ions on the first day after drug discontinuation *t* tests were made. No significant difference was found suggesting that both the sodium and the chloride loss on the first day of treatment were equal to the amount retained on the first day after drug discontinuation. Although natriuresis was obvious during the period of treatment, a slight but highly significant potassium retention was seen on the first discontinuation day (see table 3).

C. Furosamide

1 The effect of furosamide on the excretion of sodium, chloride and potassium by normal rats

After an initial 6-day control period 12 normal rats were given furosamide for 6 days followed by a second control period of 6 days. The average daily weight and the urinary excretion of sodium, chloride and potassium are shown in fig. 4. On the first day after drug administration an increase in the average excretion of sodium and chloride was observed. On the first and second day after discontinuation of furosamide administration a decrease in the excretion of these ions was observed. No change in the average daily sodium chloride excretion was seen apart from these days. The values are shown in table 4. On the first day after furosamide administration a significantly increased average potassium excretion was observed, which was maintained as long as furosamide was given. After discontinuation of treatment a decreased potassium excretion was observed. For each rat the increase in the sodium chloride and potassium excretion on the first day of treatment above the average daily control excretion was calculated. Similarly the decrease in excretion on the first and second day after drug discontinuation was calculated. The average values and the standard deviations of these six parameters were calculated. The average values for the increase in sodium and chloride excretion on the first day of treatment differed significantly when compared by a *t* test ($P < 0.001$). Similarly the sodium and chloride retention on the first 2 days after discontinuation of furosamide administration were significantly different ($P < 0.01$). The combined sodium and potassium loss on the first day of treatment and the standard deviation of this parameter was calculated. Similar calculations were made for the first and second day after drug discontinuation. The average value for the joint sodium and potassium loss and the average value for the chloride loss on the first day of treatment were compared. No significant difference was found ($P > 0.1$) indicating that the combined sodium and potassium loss was of the same magnitude as the chloride loss. Similarly the average value

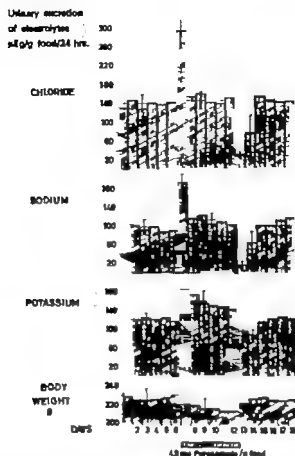


Fig. 4 Effect of furosamide medication on sodium, potassium and chloride excretion. Average values and standard deviations of 12 rats are shown. The sodium and chloride loss occurred on the first day of medication; the retention took place on the first and second day after discontinuation. A decreased potassium excretion was observed throughout the period of treatment. A weight loss was seen during the administration of furosamide.

of the sodium and the potassium retention during the first and second day after discontinuation was compared with the average chloride retention in this period. No significant difference was found ($P > 0.1$). The average values and S.D. for respectively the combined sodium and potassium loss on the first day of treatment and the retention on the first and second day after discontinuation of treatment were 158 ± 28 and $184 \pm 40 \mu\text{Eq/g food}/24 \text{ hrs.}$ These results indicate that a deficit of sodium, potassium and chloride was found on the first day after furosamide administration and maintained throughout the period and completely restored by the third day after drug discontinuation. The combined sodium and potassium

Table 4

Urinary electrolyte excretion in the furosamide experiment
 The table includes 12 rats, the units are $\mu\text{Eq/g food} / 24 \text{ hrs.}$

Average daily excretion	Control period	Chronic furosamide administration period	1 furosamide administration day	1 and 2 discontinuation day
	Mean \pm S.D.	Mean \pm S.D.	Mean \pm S.D.	Mean \pm S.D.
N	98 \pm 16	111 \pm 16 N.S.	193 \pm 21 *)	39 \pm 29)
K	121 \pm 12	154 \pm 17)	184 \pm 17)	92 \pm 9)
Cl	140 \pm 18	144 \pm 18 N.S.	292 \pm 39)	51 \pm 11)

Statistical significance from control) $0.05 > P > 0.01$) $0.01 > P > 0.001$

) $P < 0.001$ N.S. denotes not significant.

depletion was of the same magnitude as the chloride deficit. The sodium deficit was approximately 15% of the exchangeable body sodium. In agreement with this a significant average weight loss of 11 g was observed during the furosamide period of treatment ($P < 0.001$). No change in food intake was seen during the experiment.

2. The effects of furosamide administration on the osmotic concentration of the urine in the normal rat

In the experiment with the 12 rats receiving supramaximal furosamide treatment (C. 1) the daily U/P ratio and $T_{\text{H}_2\text{O}}$ values were calculated for each rat. The average daily control $T_{\text{H}_2\text{O}}$ value was compared with the average daily $T_{\text{H}_2\text{O}}$ value during the chronic period of treatment. A significant difference was found by a t-test ($P < 0.001$). In order to calculate the $T_{\text{H}_2\text{O}}$, the daily control period urine volumes were corrected to the same fractional volume loss as the furosamide period of treatment volumes.

The average daily U/P ratios are shown in fig. 2. The average control period U/P ratio was compared with the average chronic period of treatment value. A significant difference was demonstrated using a t-test ($P < 0.001$). This indicates a significant decrease in the osmotic concentration of the urine during the chronic furosamide administration period. This is in agreement with the hypothesis that chronic furosamide treatment causes a significant decrease in the concentrating ability of the normal rat kidney.

Discussion

Both thiazide and furosamide administration induce an acute salt deficit, which is maintained throughout the chronic period of medication, during which the salt excretion corresponds to the daily intake. When the drug administration is discontinued the sodium chloride deficit is restored. Thus the kidney prevents any further salt loss during the chronic drug administration period, but it does *not* compensate for the initial salt loss induced by the diuretics. The depletion is maintained unchanged. This new state most likely is due to a compensatory salt retaining mechanism located in the kidney. This means that the daily renal salt resorption during the chronic medication period is equal to the daily control renal salt resorption since no detectable changes in glomerular filtration rate were found (MORRISON 1962). Most likely a change in the fractional sodium resorption in the tubular segments has occurred. Previous investigations suggest that the main inhibition of fractional sodium resorption induced by furosamide and thiazide is located in the ascending loop of Henle and consists in an inhibition of the active salt absorption in this part of the nephron (BERLINER *et al* 1966). This would be expected to lead to a decreased concentrating ability in antidiuresis, and to a decreased diluting ability in water diuresis. The observations made in the present experiments demonstrate that during the chronic administration of a diuretic agent, concentrating ability of the kidney in antidiuresis is in fact reduced. This might be caused by a decrease in the medullary tonicity. Impaired concentrating ability was also observed during the *acute* natriuretic phase of both thiazide and furosamide treatment (KOBINGER 1965, HOOK *et al* 1965). Taken together these observations make it reasonable to assume the existence of a compensatory renal tubular salt retaining mechanism during the chronic medication period.

During chronic administration, renal sodium resorption has the same magnitude as in control periods. Is this due to unchanged fractional resorption of sodium or to decreased absorption in some renal sites and increased absorption in others? Since the concentrating ability is diminished, most likely the sodium resorption in the ascending loop of Henle is still impaired, and the medullary hypertonicity decreased (as in acute experiments). Therefore, a compensatory augmentation of the sodium resorption in other parts of the nephron will be in operation.

The observations on sodium chloride depletion made in the thiazide metabolic studies are in complete agreement with previous investigations carried out on diabetes insipidus (d.i.) rats with a similar metabolic technique as used in the present experiments (SKADHAUGE 1966).

The potassium excretion did not increase significantly during thiazide

period of treatment. Neither was this observed in the previous study (SKADHAUGE 1966). KOBINGER & KATIC (1960) however observed a significant increase in the urinary potassium excretion of normal rats receiving bendroflumethiazide in acute experiments, although the increase in the renal sodium excretion was not significantly different from the increase in urinary chloride excretion. It may be assumed that the kaliuresis produced by thiazide treatment is less pronounced in the normal rat than in human subjects.

The average daily control period sodium chloride excretion values were slightly different in the thiazide and the furosamide experiments (table 2 and 4). The difference was, however, not significant and was thus probably due to random variation.

The reason why previous studies in patients did not demonstrate any changes in extracellular fluid volume and exchangeable sodium may be that the fractional change in these values is small compared with the accuracy of the experimental method. In our study however the depletion of body salt in per cent of the average daily salt turnover is larger and therefore detectable.

The magnitude of the salt loss induced by the thiazide treatment and maintained throughout the period of drug medication in our normal rats is roughly of the same magnitude as the loss in patients reported by SANDOE & OLESEN (1962).

The slower restoration of the NaCl deficit after furosamide treatment in our experiments, could be attributed to either the larger deficit of these ions or to a more prolonged action of furosamide.

Some experiments with acute thiazide medication to normal animals in sustained salt and water diuresis have not demonstrated any significant changes in $T^0_{H_2O}$ (EARLEY *et al* 1961 SELDIN *et al* 1966). It is, however, not possible to compare acute experiments in sustained salt and water diuresis directly with our chronic experiments, which aimed at a more physiological condition corresponding to the clinical situation. Further more, when the solute excretion changes as in acute experiments, $T^0_{H_2O}$ will change even if the urine osmolality remains constant. Therefore the use of $T^0_{H_2O}$ is of limited value in estimating the "concentrating ability" under those circumstances. The constant decrease in the concentrating ability of the kidney during chronic thiazide treatment observed in our experiments is however in agreement with acute experiments made without any infusions (KOBINGER 1965) and in which a fall in urine osmolality was observed.

The fact that the salt depletion is maintained during a longterm thiazide treatment suggests that the antihypertensive action of the drug is not only due to a decrease in the peripheral resistance etc. but that it might to some extent be secondary to the salt depletion itself.

Summary

Metabolic studies on normal rats demonstrated that the acute salt depletion caused by thiazide and furosamide administration was maintained quantitatively throughout a longer period of treatment, irrespective of its length. The established salt deficit was not restored until after discontinuation of treatment, in the case of thiazide treatment on the first day and in the case of furosamide treatment, on the second day after discontinuation. During chronic supramaximal thiazide and furosamide treatment the salt deficit was 10% and 15% of extracellular body sodium, respectively. The chloride depletion was equal to the sodium depletion in the thiazide experiment and the sodium and potassium depletion in the furosamide experiment.

Both during the chronic phase of furosamide and of thiazide administration, significant decreases in the osmotic urine to plasma ratio and TC_{H_2O} were observed. Universal dehydration showed a significant decrease in the osmotic urine to plasma ratio showing that chronic thiazide treatment results in a significant decrease in the concentrating ability of the kidney.

The data seem to indicate that during the steady state phase of chronic treatment with diuretics, the fractional resorption of sodium is compensatorily increased in a part of the nephron (different from the one in which the drug decreases sodium resorption).

Acknowledgements

We are indebted to Miss Aase Frederiksen for valuable technical assistance.

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The Distribution and Urinary Excretion of Decamethonium and Hexamethonium following Intravenous Injection into Mice

By

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Preliminary studies by one of us have shown that decamethonium accumulates in the liver after intravenous injection into mice (BROES CHRISTENSEN 1967b & c). Twenty minutes after intravenous injection of 0.15 µg/g ³H-decamethonium 50% of the dose was found in the liver. Radioactivity extracted from the liver tissue with 80% methanol chromatographically corresponded to decamethonium. The significant hepatic uptake of decamethonium raised the question whether the mouse liver would accumulate hexamethonium to the same extent. MCISAAC (1962) who studied the distribution of ¹⁴C hexamethonium in cats found increasing concentrations of ¹⁴C in liver and kidneys for several hours following a single intravenous injection, the renal concentration, however being about 50 times as high as that found in the liver.

The present study was made for the purpose of comparing the distribution of hexamethonium in mice with that of decamethonium. The investigations include determinations of decamethonium and hexamethonium concentrations in the tissues 20 and 240 minutes after intravenous administration, with particular regard to the hepatic and renal uptake. As part of recovery investigations, the rates of urinary excretion of the methonium compounds were determined. Furthermore, extracts from liver were subjected to paper chromatography with regard to a possible metabolic transformation of decamethonium.

Methods

Eighty male albino mice of single strain (SVARTT) ranging in weight from 25-36 g (average 29) were used for the study. All injections were made into tail vein. 1. The first

series, 28 mice received ^3H or ^{14}C -decamethonium $0.15\text{ }\mu\text{g/g}$ body weight (injection volume $2\text{ }\mu\text{l/g}$) by means of a micrometer syringe (Aglar $\text{\textcircled{R}}$). The decamethonium dose chosen had no noticeable effect on the muscular activity of the animals. Another group of animals, the data of which are not recorded in this report, developed severe muscular weakness following intravenous injection of decamethonium $0.30\text{ }\mu\text{g/g}$.

In the second series 1152 mice received $150\text{ }\mu\text{l}$ injection solution/mouse by means of a tuberculin syringe. In this series 18 mice received ^{14}C -decamethonium $4.63\text{ }\mu\text{g/mouse}$. 34 mice were given ^{14}C -hexamethonium at two dose levels ($6.67\text{ }\mu\text{g}$ and $66.90\text{ }\mu\text{g/mouse}$). No changes in animal behaviour were observed after any of the hexamethonium injections. The low specific activity of the ^{14}C -hexamethonium preparation did not allow us to use doses of hexamethonium equimolar to that of decamethonium.

Materials

The following radiochemicals were used: ^3H -methyl-decamethonium dichloride, specific activity 128 and 139 mCi/mM ; ^{14}C -methyl-decamethonium dibromide, specific activity 5.03 and 20.9 mCi/mM ; ^{14}C -methyl-hexamethonium dichloride, specific activity 1.55 mCi/mM . The ^{14}C -decamethonium was obtained from the Radiochemical Centre, Amersham, England, the other radiochemicals from New England Nuclear Corp., Boston, U.S.A. The tritiated decamethonium preparation with high specific activity was used in the initial experiments, but as this preparation was only stable in methanol solution and liable to rapid chemical decomposition in aqueous solution, the ^{14}C -labelled decamethonium preparation was preferred for the remaining experiments. Unlabelled hexamethonium dichloride was obtained from May and Baker Ltd., and unlabelled decamethonium dibromide from Burroughs Wellcome & Co.

Preparation

The animals were stunned and bleeding produced by incision in the carotid sheath. Blood samples obtained with a heparinized Carlsberg pipette were collected in heparinized centrifuge tubes, about 0.5 ml per sample. The animals were then killed by decapitation, and the organ specimens rapidly removed. About 0.5 g liver tissue was excised, both kidneys weighing $0.3\text{--}0.5\text{ g}$ were removed and detached from their capsules. The anterior femoral muscles, weighing $2\text{--}3\text{ g}$, were carefully detached from the surrounding connective tissue.

Plasma and tissue analysis

Blood samples were centrifuged, $150\text{ }\mu\text{l}$ of the plasma was pipetted off and prepared by the method described by BROEN CHRISTENSEN (1965). The radioactivity of each sample was measured by means of a Packard Tri-Carb liquid scintillation spectrometer model 314 EX. The efficiency of the measurements was controlled by comparison with standard samples prepared by adding known amounts of ^3H or ^{14}C labelled compound to mouse plasma, which was subjected to the standard procedure. These samples showed direct proportionality between added radioactivity and counting rate (after subtraction of background) in the area of the experimental samples. The concentration of compound in the plasma expressed as μg methonium base/ml plasma was calculated from the standards. The amount of compound in the plasma volume was calculated from the mean concentration in plasma based on plasma volume of 4.4% body weight (HYM ERG, LANGGAARD, SCHOU & SZYMNY 1964). The tissue specimens were weighed immediately after excision and prepared according to the method described by BROEN CHRISTENSEN (1965). The radioactivity of each tissue specimen was measured by the same procedure as that used for the plasma

samples. The efficiency of the measurements was controlled by comparison with standard tissue specimens prepared by adding known amounts of ^3H or ^{14}C -labelled compound to tissue specimens, which were subjected to the standard procedure. These samples showed direct proportionality between added radioactivity and counting rate (after subtraction of background) in the area of the experimental samples. At the low dose level of ^{14}C hexamethonium, counting rates for muscle and organ specimens were only 2-3 times the background counting rate, but with these exceptions radioactivity measurements always gave counting rates at least 5 times the background counting rate, and not less than 5000 counts were recorded. The concentration of compound in the tissue specimens was calculated from the standards and expressed as μg methonium base/g wet weight. The percentage of the injected dose taken up by the liver and kidney was calculated from the mean concentrations in these organs, using liver weight of 5.0% body weight and a kidney weight of 1.4% body weight. In 20 animals ranging in weight from 25-35 g (average 29) we found liver weight of $5.0 \pm 0.1\%$ body weight (mean \pm S.E.M.). In 24 animals ranging in weight from 26-36 g (average 30) we found kidney weight of $1.36 \pm 0.04\%$ body weight (mean \pm S.E.M.).

Urine collection and analysis

Immediately after the injection, the animals were placed on filter papers and continuously observed until the end of the experimental period. At that time micturition was induced by suprapubic pressure, and immediately afterwards, the animals were stunned and decapitated, urine discharged during these procedures being collected by pressing the filter paper against the urethral opening. Finally the bladder was emptied as completely as possible by vigorous suprapubic compression. The wet parts of the filter papers were cut into pieces and transferred to test tubes, after which the activity was extracted by adding 4000 μl solvent (Ethanol, glacial acetic acid, water 10:1:3 V/V/V) to each sample. The solvent employed was the same as that used by BORM CHAMBERLAIN (1967a) for the extraction of ^{14}C -decamethonium from rabbit urine. The samples were shaken and stored at room temperature for about 20 minutes. A aliquot of 1000 μl was removed from each sample and pipetted into counting vials containing 8 ml scintillation medium (BRAY 1960). The radioactivity of each sample was measured by the same procedure as that employed for the plasma and tissue samples. The efficiency of the measurements was controlled by adding known amounts of ^{14}C -labelled compound to filter papers previously moistened with mouse urine and subjecting them to the procedure described. These samples showed direct proportionality between added radioactivity and counting rate (after subtraction of background) in the area of the experimental samples. The amount of methonium base excreted was calculated from a standard curve and expressed as percentage of the dose injected. The extraction method applied yielded recovery of $100.8 \pm 1.4\%$ for beta-methonium and $104.1 \pm 2.3\%$ for decamethonium (mean \pm S.E.M. of values from 8 experiments).

Preparation of chromatograms

Liver deproteinates obtained by the procedure previously described for tissue analysis were chromatographed by an ascending technique on Whatman paper no. 1. Two different systems were used as the mobile phase: A, n-butanol, ethanol, glacial acetic acid, water (8:2:1:3); B, pyridine, ethanol, water (1:1:1). ^{14}C -decamethonium added to blank tissue specimen which was carried through the standard procedure served as reference. The ^{14}C activity was scanned with an automatic radiochromatogram scanner Packard (model 7201).

Table 1

Decamethonium concentrations in plasma ($\mu\text{g/ml}$) and tissues ($\mu\text{g/g}$) within 20 minutes following i.v. injection of ^3H or ^{14}C -decamethonium 0.15 $\mu\text{g/g}$.

Results are given as the mean \pm S.E.M. of values from 6-8 experiments. Percentage of injected dose in plasma and tissues was calculated from the mean concentrations and the following values: plasma volume 4.4%, muscular mass 50% and liver 5.0% body weight (cf. text).

Tissues	Plasma			Striated muscles	Liver	Liver
min after inj.	5	10	20	20	20	20
Mean conc.	0.28	0.08	0.032	0.043	1.54	1.41
= S.E.M.	± 0.06	± 0.01	± 0.003	± 0.004	± 0.09	± 0.03
Per cent of dose	2.2	0.4	0.9	14	51	48
Isotope	H					^{14}C

Results

Distribution of decamethonium.

The results of decamethonium determinations in plasma and tissues within 20 minutes following intravenous injection of ^{14}C or ^3H -decamethonium 0.15 $\mu\text{g/g}$ are shown in table 1. The decamethonium concentration in plasma shows a rapid decrease and less than 1% of the dose is found in the plasma volume 20 minutes after injection. The decamethonium concentration in the striated muscles is somewhat higher than the plasma concentration. We have no information about the muscular mass of mice, but calculating with a value of 50% body weight, the decamethonium uptake in the striated muscles constitutes 14% of the injected dose. The liver contains decamethonium at a concentration about 50 times as high as that found in plasma. Approximately 50% of the dose accumulates in the liver 20 minutes after injection of ^3H or ^{14}C -decamethonium, the difference between results obtained with the ^3H and the ^{14}C -compound being negligible.

Table 2 shows the results of decamethonium determinations in liver and kidney 20 minutes after the administration of ^{14}C -decamethonium 4.63 $\mu\text{g/mouse}$ (average 0.14 $\mu\text{g/g}$). The recovery of decamethonium from the liver in this series of experiments is somewhat lower than that found in the earlier experiments reported above. The dose was nearly the same in both series. The highest concentration of decamethonium

Table 2

Decamethonium concentrations ($\mu\text{g/g}$) in liver and kidney 20 min. after i.v. injection of ^{14}C -decamethonium ($4.63 \mu\text{g/mouse}$).

Results are given as the mean \pm S.E.M. of values from 6 experiments. Percentage of injected dose in liver and kidney was calculated from the mean concentrations and the following values: liver 5.0% and kidney 1.4% body weight (cf. text).

Tissue	Liver	Kidney
Mean conc. \pm S.E.M.	1.12 ± 0.06	1.72 ± 0.10
Per cent of dose	41	18

) Mean animal weight 34 g (range 31–35).

was found in the kidney approximately 18% of the dose being present in this organ.

Distribution of hexamethonium

^{14}C -hexamethonium was given at two dose levels. Group A received $6.67 \mu\text{g/mouse}$ (average $0.22 \mu\text{g/g}$) and group B received $66.90 \mu\text{g/mouse}$ (average $2.38 \mu\text{g/g}$).

Table 3

^{14}C -hexamethonium concentrations in plasma ($\mu\text{g/ml}$) and tissues ($\mu\text{g/g}$) 20 minutes after i.v. injection of hexamethonium at two dose levels, A $6.67 \mu\text{g/mouse}$, B $66.69 \mu\text{g/mouse}$.

Results are given as the mean \pm S.E.M. of values from 4–8 experiments. Figures in brackets indicate percentage of injected dose in plasma and tissues, calculated from the mean concentrations and the following values: plasma volume 4.4%, liver 5.0%, kidney 1.4% and muscular mass 50% body weight (cf. text).

Tissue	Plasma	Liver	Kidney	Striated muscles
A (Dose, $6.67 \mu\text{g/mouse}$)	0.17 ± 0.01 (3.3)	0.21 ± 0.03 (4.7)	0.76 ± 0.05 (4.8)	0.025 ± 0.003 (5.7)
B (Dose, $66.90 \mu\text{g/mouse}$)	1.11 ± 0.14 (3.9)	1.62 ± 0.12 (3.4)	2.71 ± 1.20 (3.1)	0.34 ± 0.06 (7.1)

*) Mean animal weight 30 g (range 28–34). **) Mean animal weight 28 g (range 26–29).

Table 1

Decamethonium concentrations in plasma (ng/ml) and tissues ($\mu\text{g/g}$) within 20 minutes following i. v. injection of ^3H or ^{14}C -decamethonium 0.15 $\mu\text{g/g}$.

Results are given as the mean \pm S.E.M. of values from 6-8 experiments. Percentage of injected dose in plasma and tissues was calculated from the mean concentrations and the following values: plasma volume 4.4%, muscular mass 50% and liver 5.0% body weight (cf. text).

Tissues	Plasma			Striated muscles	Liver	Liver
min. after inj.	5	10	20	20	20	20
Mean conc.	0.28	0.08	0.032	0.043	1.54	1.41
\pm S.E.M.	± 0.06	± 0.01	± 0.003	± 0.004	± 0.09	± 0.08
Per cent of dose	8.2	2.4	0.9	14	51	48
Isotope	^3H				^{14}C	

Results

Distribution of decamethonium.

The results of decamethonium determinations in plasma and tissues within 20 minutes following intravenous injection of ^{14}C or ^3H -decamethonium 0.15 $\mu\text{g/g}$ are shown in table 1. The decamethonium concentration in plasma shows a rapid decrease and less than 1% of the dose is found in the plasma volume 20 minutes after injection. The decamethonium concentration in the striated muscles is somewhat higher than the plasma concentration. We have no information about the muscular mass of mice, but calculating with a value of 50% body weight, the decamethonium uptake in the striated muscles constitutes 14% of the injected dose. The liver contains decamethonium at a concentration about 50 times as high as that found in plasma. Approximately 50% of the dose accumulates in the liver 20 minutes after injection of ^3H or ^{14}C -decamethonium the difference between results obtained with the ^3H and the ^{14}C -compound being negligible.

Table 2 shows the results of decamethonium determinations in liver and kidney 20 minutes after the administration of ^{14}C -decamethonium 4.63 $\mu\text{g/mouse}$ (average 0.14 $\mu\text{g/g}$). The recovery of decamethonium from the liver in this series of experiments is somewhat lower than that found in the earlier experiments reported above. The dose was nearly the same in both series. The highest concentration of decamethonium

Table 5

Retention of decamethonium and hexamethonium in liver and kidney 240 minutes after I. Injection

Dose ^{14}C -decamethonium, 4.63 $\mu\text{g}/\text{mouse}^*$ ^{14}C hexamethonium 6.67 $\mu\text{g}/\text{mouse}$).

Results are given as the mean \pm S.E.M. of values from 7-8 experiments. Percentage of injected dose in liver and kidney was calculated from the mean concentrations and the following values: liver 5.0% and kidney 1.4% body weight (cf. text).

Tissue Compound	Liver		Kidney	
	Decamethonium	Hexamethonium	Decamethonium	Hexamethonium
Concentration ($\mu\text{g}/\text{g}$)	1.40 ± 0.07	0.09 ± 0.1	1.15 ± 0.05	0.29 ± 0.03
Percent of dose	42	19	10	17

) Mean animal weight 28 g (range 26-30).) Mean animal weight 28 g (range 25-30)

Retention of decamethonium and hexamethonium in liver and kidney

Table 5 shows the results of determinations of methonium concentrations in liver and kidney 240 minutes after the intravenous administration of ^{14}C -decamethonium 4.63 $\mu\text{g}/\text{mouse}$ (average 0.17 $\mu\text{g}/\text{g}$) or ^{14}C hexamethonium 6.67 $\mu\text{g}/\text{mouse}$ (average 0.24 $\mu\text{g}/\text{g}$) 42% of the decamethonium dose was found in the liver which is approximately the same percentage uptake as 20 minutes after the injection, cf. table 2. 240 minutes after the injection the decamethonium concentration in the liver was higher than in the kidney the amount of decamethonium in the kidney being reduced from 18% to 10% of the dose during the period 20 to 240 minutes after the injection. During the same period the content of hexamethonium in the liver as well as in the kidney was reduced by 60-65% (cf. table 3) 240 minutes after the injection more than 50% of the decamethonium dose was still retained in the liver and kidneys, whereas less than 4% of the hexamethonium dose was recovered from these organs.

Paperchromatographic results

Deproteinates from liver specimens, excised 240 minutes after intravenous injection of ^{14}C -decamethonium, were subjected to paper chromatography. Chromatograms with two different systems contained only a single spot of radioactivity corresponding in R_f to authentic decamethonium.

Discussion

The present results demonstrate considerable differences between decamethonium and hexamethonium regarding their distribution and elimination in mice. Thus, 20 minutes after the injection 60% of the decamethonium dose was taken up by the liver and kidney whereas less than 10% of the hexamethonium injected was found in these organs. Within the same period following the injection 60% of the hexamethonium dose was excreted in the urine, while the excretion of decamethonium only constituted 13-15% of the dose.

The rapid fall of radioactivity in plasma following intravenous injection of labelled decamethonium, also observed by WASER (1963) is most likely due to the significant hepatic and renal accumulation of this compound.

The concentration of hexamethonium in the striated muscles suggests an extracellular distribution of this agent, whereas the muscular uptake of decamethonium which greatly exceeds the capacity of the extracellular space is consistent with a specific accumulation, as shown by WASER & LÜTHI (1957).

The decamethonium uptake in the liver of mice following intravenous administration greatly exceeds that found in rabbits (BROEN CHRISTENSEN 1965) and in cats (WASER 1965). In an unpublished series of experiments we administered 0.15 µg/g ³H-decamethonium intravenously to 8 rats. Twenty minutes after injection, the average hepatic concentration was 0.16 µg/g, which meant that about 2.5% of the dose was taken up by the liver. Recent investigations (BROEN CHRISTENSEN 1967b & c) into the mechanism of decamethonium accumulation in slices of mouse liver have shown that the process is energy dependent which is indicative of a penetration of decamethonium into the liver cells. Our demonstration of a decamethonium content in the liver 240 minutes after the administration, which is practically the same as that found initially makes biliary excretion as a major pathway of elimination unlikely. Our chromatographic studies show that decamethonium is not metabolized in the mouse liver. The hexamethonium uptake in the liver of mice is low which is also the case in cats (McISAAC 1962) and rats (LEVINE 1960). LEVINE furthermore showed that hexamethonium was not metabolized in mice.

Decamethonium as well as hexamethonium is concentrated at the highest level in the kidneys, the uptake of decamethonium, however exceeding that of hexamethonium by 3 to 4 times. McISAAC (1962) found particularly high ¹⁴C-concentrations in the kidneys following intravenous injection of ¹⁴C-hexamethonium into cats. Autoradiographic studies showed that the radioactivity was primarily localized in the tubule

cells of the cortex. *In vitro* studies (McISAAC 1965) have also shown an excessive renal accumulation of hexamethonium in cats, whereas the concentration of hexamethonium in kidney slices from rabbits and rats did not exceed that of the medium. The rapid renal excretion of hexamethonium in mice might suggest a tubular secretion of this compound but the unchanged rate of excretion after a tenfold higher dose does not support this hypothesis. The significantly different rates of urinary excretion for decamethonium and hexamethonium are most likely due to the different distributions and consequently different plasma levels of these agents. Investigations of the renal elimination of methonium compounds in other mammals suggest an excretion primarily involving glomerular filtration. The renal clearance of hexamethonium in cats has been determined by McISAAC (1962) and decamethonium clearance in rabbits by BROEN CHRISTENSEN (1965). McISAAC (1965) has suggested that an active process is important for the accumulation of hexamethonium in the cat kidney and that the renal tubular transport system for organic bases may participate in the uptake of hexamethonium, which is subsequently bound to nonspecific sites intracellularly. The very high renal uptake of decamethonium in our experiments might indicate an uptake by similar tubular mechanisms in the mouse, but our *in vivo* results do not allow of such an interpretation. *In vitro* studies of the accumulation of decamethonium in kidney tissue from mice are necessary to elucidate the uptake mechanism.

Summary

An account is given of the distribution and urinary excretion following intravenous injection of decamethonium and hexamethonium into mice.

The results demonstrate considerable differences between the distribution and elimination of these two methonium compounds. Thus, about 60% of the decamethonium dose was taken up by liver and kidney within 20 minutes after the injection, whereas less than 10% of the hexamethonium injected was found in these organs. Within the same period about 60% of the hexamethonium dose was excreted in the urine.

The decamethonium content in the liver 20 and 240 minutes after the injection is practically the same. The radioactivity extracted corresponded chromatographically to authentic decamethonium.

The mechanisms involved in the high renal and hepatic accumulation of decamethonium as well as the renal elimination of methonium compounds are discussed.

Acknowledgements

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Studies on Riboflavin Metabolism in the Rat. I. Urinary and Faecal Excretion after Oral Administration of Riboflavin-5'-phosphate

By

Sten Christensen

(Received September 11 1968)

The relation between the dose of riboflavin administered orally and the urinary recovery of the vitamin in man was determined in this laboratory by STRIPP (1965) who showed that doses above 50 mg FMN (flavin mononucleotide, riboflavin-5' phosphate) did not cause any further excretion of riboflavin. This observation was confirmed by LEVY & JUSKO (1966) who also showed that ingested FMN is excreted in the same way as if riboflavin had been given (JUSKO & LEVY 1967).

In order to obtain a better understanding of the apparently capacity-limited absorption of riboflavin, STRIPP carried out experiments in rats which suggested an upper limit for the riboflavin excretion as in man (STRIPP unpublished results). This study has been continued and in the present paper findings are presented on urinary and a faecal excretion after oral doses of up to 4 mg FMN to rats.

Methods

Female Wistar rats with an average weight of 255 g (range 233-288 g) were kept in pairs in modified metabolic cages (fig. 1) which permitted the collection of urine and faeces separately. The rats were given 15 g of riboflavin deficient diet¹⁾ daily which kept their body weight constant during the experiment. Preliminary experiments showed that the flavin excretion in the urine and faeces had practically stopped 72 hours after FMN administration, regardless of the dose given. Each pair of rats received 4 or 5 doses, beginning with small doses and with an interval of 14 days between the administrations. The sodium salt of FMN dissolved in 1 ml water was administered by stomach tube.

¹⁾ From Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

FIG. 1

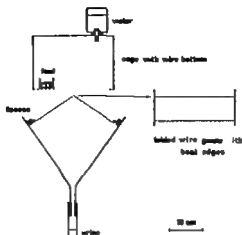


Fig. 1 Metabolic cage for separate collection of urine and feces. Feces are removed by roof-shaped wire gauze with bent edges. The food is mixed with water to prevent loss through the bottom of the cage. The urine collecting container is light-imperious. Mesh sizes: Cage bottom 12 mm and wire gauze 4 mm.

Analytical methods Urine samples were diluted to 50 ml with water. Feces were homogenized with an "Ultra Turax" homogenizer type 18/2 after addition of 20 ml water and water was added to give a total volume of 50 ml. The diluted samples were filtered through paper No. 540. No desorption of the vitamin in the paper was found.

Thin-layer chromatography (pyridine-glacial acetic acid-water 10:1:40 on 0.25 mm silica gel layer) of the samples showed only one fluorescent spot, corresponding to free riboflavin and no traces of FMN or FAD so that the vitamin in the urine and feces was present only as riboflavin.

Standard solutions were prepared in suitable concentrations by dilution of a concentrated aqueous solution of riboflavin. Tests, standard solutions and blanks consisting of water were prepared as follows:

100 μ l were transferred into a test tube with 1 ml of 10% trichloroacetic acid. After centrifugation 0.5 ml of the supernatant was mixed with 0.5 ml 1 M K₂HPO₄ in a cuvette, and the fluorescence of the neutralized solution was measured in a Photovolt filter fluorometer with "HQ 3" as primary and B 340" as a secondary filter. 25 μ l of 10% Na₂S₂O₅ in 5% NaHCO₃ were added for reduction and after stirring the fluorescence of the solution was measured again. The riboflavin concentration is proportional to the difference between the two readings, corrected for dilution with the reduction reagent. The absolute content of riboflavin was calculated from the values obtained by the test, the standard and the blank solutions and the dilution factor.

The recovery of riboflavin, when added to diluted samples, was more than 96% for urine and more than 93% for feces. The method is a modification of that described by BURCH *et al.* (1948).

Results

The urinary excretion of riboflavin after administration of 0-4000 μ g FMN is shown in tables 1 and 2, which list the excretion after

Table 1

Urinary excretion of riboflavin 0-24 hours after oral administration of FMN to rats.
Each group represents two rats. μg riboflavin excreted/rat.

Group	Dose of FMN μg riboflavin equivalent					
	0	200	500	1000	2000	4000
A	2.1	28.1	32.0	70.0	39.0	58.3
B	4.5	35.3	38.5	47.4	108.4	113.5
C	3.9	2.5	24.8	28.9	90.8	74.7
D	3.5	1.5	22.5	50.0	-	-
Mean	3.5	16.9	29.5	49.1	79.4	82.2
Recovery i.e. minus basal excretion		13.4	26.0	35.6	75.9	78.7
Recovery %		6.7	5.2	4.6	5.8	2.0

24 and 72 hours, respectively. The mean values are plotted against the dose and the best fitted curves are shown in fig. 2. The lower curve shows the 24-hour excretion and the upper curve the 72-hour excretion. The curves show a rectilinear relationship between dose and excretion in dose ranges of 0-1000 and 0-2000 μg , respectively. The slopes are 0.044 for the 24-hour excretion and 0.051 for the 72-hour excretion signifying that 4.4 and 5.1 %, respectively of the dose administered were excreted in the urine. However the difference is not statistically significant. With doses above 1000 (2000) μg the amount excreted in per cent of the dose decreased. The amount excreted in the first 24 hours seems to become

Table 2

Urinary excretion of riboflavin 0-72 hours after oral administration of FMN to rats.
Each group represents two rats. μg riboflavin excreted/rat.

Group	Dose of FMN μg riboflavin equivalent					
	0	200	500	1000	2000	4000
A	6.7	35.2	48.2	90.0	59.3	110.9
B	11.3	37.9	50.2	54.2	166.7	212.1
C	12.8	7.7	33.2	36.9	106.1	126.0
D	12.9	4.9	30.9	57.6	-	-
Mean	10.9	21.4	40.6	60.2	114.0	149.7
Recovery i.e. minus basal excretion		10.5	29.7	49.3	103.1	138.8
Recovery %		5.3	5.9	4.9	5.2	3.5

FIG. 1

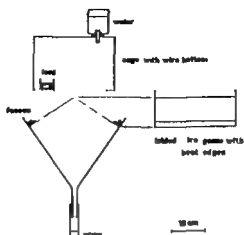


Fig. 1 Metabolic cage for separate collection of urine and faeces. Faeces are removed by a roof-shaped wire gauze with bent edges. The food is mixed with water to prevent loss through the bottom of the cage. The urine collecting container is impervious to light. Mesh sizes: Cage bottom 12 mm and wire gauze 4 mm.

Analytical methods Urine samples were diluted to 50 ml with water. Faeces were homogenized with an Ultra Trazor homogenizer type 18/2 after addition of 20 ml water and water was added to give total volume of 50 ml. The diluted samples were filtered through paper. No adsorption of the vitamin to the paper was found.

Thin-layer chromatography (pyridine-glacial acetic acid-water 10:1:40 on a 0.25 mm silica gel layer) of the samples showed only one fluorescent spot, corresponding to free riboflavin and no traces of FMN or FAD so that the vitamin in the urine and faeces was present only as riboflavin.

Standard solutions were prepared in suitable concentrations by dilution of a concentrated aqueous solution of riboflavin. Tests, standard solutions and blanks consisting of water were prepared as follows:

100 μ l were transferred into a test tube with 1 ml of 10% trichloroacetic acid. After centrifugation 0.5 ml of the supernatant was mixed with 0.5 ml 1 M K_2HPO_4 in a cuvette and the fluorescence of the neutralized solution was measured in a Photovolt filter fluorometer with HG 3 as primary and B 340 as a secondary filter. 25 μ l of 10% $N_2S_2O_4$ in 5% NH_4CO_3 were added for reduction and after stirring the fluorescence of the solution was measured again. The riboflavin concentration is proportional to the difference between the two readings, corrected for dilution with the reduction reagent. The absolute content of riboflavin was calculated from the values obtained by the test, the standard and the blank solutions and the dilution factor.

The recovery of riboflavin, when added to diluted samples, was more than 96% for urine and more than 93% for faeces. The method is a modification of that described by BURCH *et al.* (1948).

Results

The urinary excretion of riboflavin after administration of 0–4000 μ g FMN is shown in tables 1 and 2, which list the excretion after

excreted between 24 and 72 hours after the ingestion as against 43% after administration of 4000 μ g.

The faecal excretion of riboflavin after administration of up to 1000 μ g FMN is shown in table 3. A rectilinear relationship was found between dose and excretion. The slope of the best fitted curve is 0.124 indicating that about 12% of the dose was excreted with the faeces.

Discussion

Qualitative relations

In spite of the fact that the vitamin was administered as the FMN-salt, only free riboflavin was found in the urine. This is in agreement with unpublished observations in rats by STRIPP whereas YAGI *et al* (1966) found that 3% of 14 C riboflavin given subcutaneously to rats was excreted in the urine as FMN. In man only free riboflavin is found in the urine after oral or intravenous administration of riboflavin or FMN (STRIPP 1965; JUSKO & LEVY 1967).

Only free riboflavin was found in the faeces in the present experiments. This is in agreement with the experiments of OKUDA (1958a & b) who found a very rapid dephosphorylation of FMN by *in vitro* incubation with pancreatic juice, total small intestine or small intestine mucosa from the dog. Previous experiments (CHRISTENSEN 1969) also showed a rapid dephosphorylation of FMN when incubated with intestinal juice or homogenized mucosa from the small intestine of the rat, whereas free riboflavin was not decomposed under these conditions. OKUDA (1958b) found the dephosphorylation in mucosa homogenate to be pH-dependant and inhibited by EDTA, by pyrophosphate and by orthophosphate which made him assume that dephosphorylation is caused by alkaline phosphatases or specific enzymes in the small intestine. The excretion of free riboflavin after oral administration of FMN may thus be explained as a result of an enzymatic dephosphorylation in the intestinal tract.

Quantitative relations

STRIPP (1965) in man found a rectilinear relationship between the oral dose of FMN and the urinary excretion of riboflavin after doses up to 30 mg, the urinary recovery being 33% of the dose. Doses over 50 mg caused no further increase. JUSKO & LEVY (1967) obtained similar results, the recovery however being 63% of the dose in the rectilinear range. This is in accordance with the results of MORRISON & CAMPBELL (1960) who in the dose range of 1–20 mg riboflavin, found a urinary recovery of 61%. However both experiments showed that in man the

urinary excretion is limited, so that oral doses over 50 mg do not cause a further increase of the excretion. This has led to the assumption that the absorption of riboflavin by man is a capacity limited process (STRIPP 1965 LEVY & JUSKO 1966)

Several investigators have measured urinary excretion in riboflavin-deficient rats or in rats receiving small amounts of riboflavin but not after the administration of large doses. In the present study a rectilinear relationship was found between dose and excretion in a large dose range, about 5 / of the dose being recovered in the urine. Compared with the recovery of 33-60 / in man, the amount excreted in the rat is small. Furthermore, there appears to be no upper limit for the excretion as found in man. Admittedly a tendency of the excretion curves to level off is seen (fig. 2) but no definite level can be observed and the levelling only appears after massive doses, namely 8 mg/kg as compared with 0.5 mg/kg in man.

About 12 / of the dose appeared in the faeces. The total excretion in the urine and faeces thus amounts to only 17 / of the dose ingested. The fate of the remaining larger part of the dose in the rat is unknown and is the subject of further studies.

Summary

- 1 After oral administration of FMN to rats, only free riboflavin was detected in the urine and faeces.
- 2 In the dose range of 0-2000 μ g the urinary excretion was found to be proportional to the dose of FMN. The urinary recovery was 5 / of the dose.
- 3 In the dose range of 0-1000 μ g the faecal excretion of riboflavin was found to be proportional to the dose of FMN. The faecal recovery was 12 / of the dose.
- 4 Unlike the results obtained in man the present results do not, within a reasonable dose range, indicate the presence of an upper limit for the urinary excretion of orally administered riboflavin.

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Studies on Riboflavin Metabolism in the Rat. II. Metabolic Flavin Elimination after Oral or Intraperitoneal Administration of Riboflavin-5'-phosphate

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After the oral administration of large amounts of FMN (riboflavin-5-phosphate) to rats only about 17% of the dose was found to be excreted in the urine and faeces (CHRISTENSEN 1969). In the present paper a study of the fate of the remaining part of the ingested dose is described.

Methods

Female Wistar rats weighing about 150 g and fed on normal diet were used in the following experiments

1. Unfasted rats received 1000 µg FMN/100 g bodyweight or about 0.75 ml of an aqueous solution of 2 mg FMN/ml by mouth tube. During the experiment they were given a riboflavin-deficient diet (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.), and the urine and faeces were collected in metabolic cages previously described (CHRISTENSEN 1969). Zero, 1, 3, 7, 24 and 72 hours after the ingestion 4 rats were killed by blow on the neck and depilated by depilatory powder (Wheat flower 350 g, Talcum 350 g, BaS (yellow) 250 g, and Soap powder 50 g) mixed with water. The entire digestive tract was ligated and removed. The body was ground in meat grinder water added and the mixture homogenized with an Ultra Turax homogenizer type T 45 with addition of more water to total volume of 1.5 l. Two hundred ml ethanol were added to prevent foaming and the volume was made up to 2 l with water. After shaking, smaller part of the homogenate was finely homogenized with an Ultra Turax homogenizer type 18/2. The digestive tract was homogenized in the same way and made up to total volume of 100 ml with water. The total flavin contents of the homogenates were determined as previously described (CHRISTENSEN 1969).

2. In the second experiment 1000 µg FMN/100 g rat were injected intraperitoneally. The rats were killed 0, 1, 2, 7 or 24 hours after the injection and treated as described above, except that the intestinal tract was not removed.

3. The small intestine of a rat was removed, rinsed with water and opened lengthwise. The mucosa layer was scraped off with a plastic scraper and homogenized with 15 ml water with an "Ultra Turax" homogenizer type 18/2. The pH of the homogenate was adjusted to 7.0 by addition of 2 M K_2HPO_4 . To 1 ml homogenate was added 1) 1 ml riboflavin or FMN solution 2) 0.2 ml water or 0.1 M EDTA and the mixture was incubated at 37° for 1 hour. After incubation 0.1 ml of the mixture was added to 5 ml 10% trichloroacetic acid and riboflavin and the FMN was determined separately as described by STAUFF (1965).
4. The small intestine of a rat was removed and the intestinal juice washed out with water (a volume of 10 ml) and filtered. To 1 ml of filtrate were added 1) 1 ml riboflavin or FMN solution 2) 0.2 ml water or 0.1 M EDTA and the mixture was incubated at 37° for 21 hours. After incubation for 1 or 21 hours, samples of 0.1 ml were added to 5 ml 10% trichloroacetic acid, and separate analyses for riboflavin and FMN were carried out as described by STAUFF (1965).
5. The digestive tract of a rat was removed and the contents of the entire intestine including the caecum were washed out with water, homogenized and water added to a volume of 30 ml. 0.1 ml of aqueous solution (2 mg FMN/ml) was added to 20 ml of the homogenate and the mixture was incubated at 37°. Zero, 1, 2, 4, 6 and 24 hours after the addition, 0.1 ml samples were added to 5 ml 10% trichloroacetic acid, and the total flavin analyses were carried out as described below. To 5 ml of incubation mixture were added streptomycin and tetracycline in final concentrations of 6 mg/ml and samples for analysis were taken after 0 and 24 hours.

Determination of total flavins in homogenate

100 μl homogenate were added to 1 ml 10% trichloroacetic acid, and the test tube stoppered with rubber plug and stored for 20 hours at 37° or for 3 days at room temperature. After the hydrolysis the tube was centrifuged, and 0.5 ml of the supernatant added to 0.5 ml 1 M K_2HPO_4 in a cuvette. The fluorescence of the neutralized solution was measured as described previously (CHRISTENSEN 1969). The recovery of riboflavin added to minced rats was more than 95%.

Results

Fig. 1 shows the results of the oral administration of FMN (experiment 1). The amount of flavin in the body minus the digestive tract was not increased. There even seems to have been a small decrease in the body flavin 24 and 72 hours after the administration. The amount of flavin in the whole body decreased rapidly after the administration. After 7 hours half of the flavin administered was eliminated, and the basal tissue concentration was reached after 24 hours. At this time 7% of the dose was found in the urine, 22% in the faeces and none in the body. The remaining 71% of the dose could not be recovered as riboflavin.

Fig. 2 shows the results obtained after intraperitoneal injection of FMN (experiment 2). Half of the flavin injected was eliminated from the body within 1 hour and the basal tissue concentration was reached after 24 hours, when 29% of the dose was recovered in the urine, 8% in the faeces and none in the body. The remaining 63% of the dose could not be recovered as riboflavin.

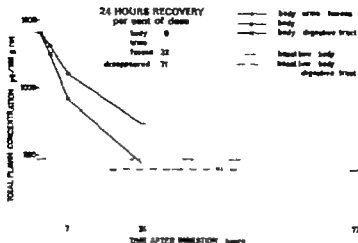


Fig. 1. Total flavin contents in the rat, urine and faeces 0-72 hours after oral administration of 1000 µg FMN/100 g rat. Each point and the basal lines represents the average of 4 rats.

From tables 1 and 2 (experiments 3 and 4) it is seen that no decrease in the total amount of flavin was observed after incubation with mucosa homogenate or juice from the small intestine. FMN was dephosphorylated to free riboflavin and the dephosphorylation rate was altered by addition of EDTA increased in the mucosa homogenate and decreased in the intestinal juice.

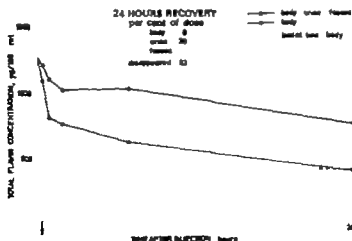


Fig. 2. Total flavin contents in the rat, urine and faeces 0-24 hours after intraperitoneal injection of 1000 µg FMN/100 g rat. Each point and the basal line represents the average of 2 rats.

Table 1

The influence of small intestinal mucosa homogenate on riboflavin and FMN. The columns indicate the concentration in the incubation mixture, $\mu\text{g/ml}$ riboflavin equivalent. Means of duplicate assays.

Incubation mixture	Before incubation			After 1 hour 37		
	RIB	FMN	Total	RIB	FMN	Total
Riboflavin	22.7	0	22.7	22.4	0.3	22.7
Riboflavin + 0.01 M EDTA	22.7	0	22.7	22.3	0.3	22.6
FMN	0	22.7	22.7	17.4	5.1	22.5
FMN + 0.01 M EDTA	0	22.7	22.7	19.6	3.1	22.7

Table 2

The influence of small intestinal juice on riboflavin and FMN. Concentrations in the incubation mixture, μg riboflavin equivalent. Means of duplicate assays.

Incubation mixture	Before incubation			After 1 hour 37			After 21 hours, 37		
	RIB	FMN	Total	RIB	FMN	Total	RIB	FMN	Total
Riboflavin	22.7	0	22.7	22.5	0	22.5	22.6	0	22.6
- + 0.01 M EDTA	22.7	0	22.7	22.6	0	22.6	22.7	0	22.7
FMN	0	22.4	22.4	20.3	2.0	22.3	21.3	1.0	22.3
FMN + 0.01 M-EDTA	0	22.4	22.4	9.4	13.0	22.4	16.9	5.5	22.4

Table 3

The influence of homogenate of contents from the whole intestine including the caecum on FMN. Concentrations in the incubation mixture, $\mu\text{g/ml}$ riboflavin equivalent. Means of duplicate assays.

	Incubating time, hours						% destroyed after 24 hours
	0	1	2	4	6	24	
Total flavin concentration	10.5	9.7	9.5	9.6	9.6	9.0	14
Total flavin concentration with tetracycline and streptomycin added	10.5					9.4	10

Table 3 (experiment 5) shows a decrease of 14% of the added flavin or about 30 µg by incubation with contents from the whole intestine for 24 hours. No marked inhibition of the destruction was observed when antibiotics were added.

Discussion

Most of the flavin administered in experiments 1 and 2 could not be recovered as riboflavin and must therefore have been destroyed in the rat organism. The fact that the destruction occurred to the same extent after oral or intraperitoneal administration suggests that this was due to metabolic processes in the organism. Moreover the much more rapid rate of disappearance after intraperitoneal administration supports the view that the metabolic elimination after oral administration is delayed by the absorption from the intestine.

The fact that 8% of the dose following intraperitoneal injection was recovered in the faeces indicates that excretion into the intestine occurred. This is in accordance with the findings of SELYE (1943) who observed excretion of riboflavin into the upper small intestine after the intravenous infusion of 7 mg riboflavin into nephrectomized rats.

Experiments 3, 4 and 5 support the assumption that the major destruction of ingested flavin takes place in the organism. No destruction was observed by incubation with the preparations of the small intestine, and only a small part of the flavin was decomposed by incubation with the total intestinal contents, presumably through the action of the local bacterial flora.

Metabolic decomposition of large amounts of flavin in the rat or man has not been investigated previously. BESSEY *et al* (1958) determined the rate of metabolic elimination in riboflavin-deficient rats by estimating the amount of riboflavin that must be supplied to non-growing rats to maintain their tissue concentration at a given level. In these conditions the metabolic rate was found to be 0.04 µg/g rat/day. FAULKNER & LAMBOOY (1961) used young riboflavin deficient rats with ¹⁴C riboflavin incorporated into their tissues and calculated the rate of metabolic elimination from the disappearance of tissue ¹⁴C-activity. They found a rate of 1.26 µg/rat/day, well in agreement with the results of BESSEY *et al*.

The relationship between the flavin metabolism and the caloric metabolism was studied by BESSEY *et al* (1958). An increase of the caloric metabolism by thyroxine or changes in the diet did not alter the rate of the flavin metabolism, suggesting that the latter is not associated with its action as a vitamin. This is in agreement with the fact that some riboflavin

analogues (ω -hydroxyalkylisoalloxazines) which have no vitamin effect *per se* possess a sparing effect of riboflavin when administered simultaneously with the vitamin to growing rats (OGUNMODEDE & MCCORMICK 1966). This action may be explained as a displacement of riboflavin from receptors in the metabolic enzyme system, so that the vitamin is protected from metabolic destruction.

The present study shows that the dietically balanced rat is not able to retain riboflavin. Administration of large doses of FMN caused rather a decrease in the total body flavin content. BESSEY *et al* (1958) showed that the tissue flavin of young rats varies only between narrow limits, i.e. 2.00–3.25 $\mu\text{g/g}$ liver free carcass corresponding to the administration of 0–40 μg riboflavin/day. Greater quantities than 40 μg daily caused no further increase in the tissue flavin. On the other hand, insufficient administration could not decrease the tissue flavin below a minimum level, the decrease being compensated for by a loss in body weight.

The unavoidable metabolism of ingested flavin is in agreement with the lack of storage capacity and may explain the observation by MORRISON *et al* (1961) that divided doses of riboflavin are better utilised in growing rats than the equivalent single doses.

Summary

- 1 The fate of large oral and intraperitoneal doses of riboflavin-5 phosphate (FMN) in rats has been studied. 24 hours after the administration, all the administered flavin was eliminated from the body and only about one third was recovered in the urine and faeces. After intraperitoneal administration some of the dose was recovered in the faeces indicating its secretion into the intestine.
- 2 No destruction of flavin could be observed by *in vitro* incubation with intestinal mucosa or with intestinal juice from rats. A rapid dephosphorylation of FMN to free riboflavin was found in both cases.
- 3 A slight destruction of flavin could be observed by *in vitro* incubation with contents from the entire intestinal tract.
- 4 It is suggested that flavin administered to rats is metabolized rapidly by a mechanism which has no connection with its vitamin activity. The metabolic elimination explains the lack of storage capacity for flavin in the rat.

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Studies on Riboflavin Metabolism in the Rat. III.

Disappearance Rate and Urinary Excretion Rate of Flavin after Intravenous Infusion of Riboflavin-5'-phosphate

Stein Christensen

By

(Received September 11 1968)

After the oral or intraperitoneal administration of large amounts of FMN (riboflavin-5 phosphate) to rats, the flavin is eliminated from the body within 24 hours (CHRISTENSEN 1969b). Part of the dose is excreted but the main part appears to be metabolized in the organism. In this paper the determination of the flavin disappearance rate from the blood and the urinary excretion rate, after intravenous infusion of FMN are detailed

Methods

1. Male Wistar rats weighing about 300 g were anaesthetized with m-bumal-sodium (Nembutal®), 50 mg/kg p., and fixed to heated surgical table the rectal temperature was kept between 37 and 39°. A polyethylene tube with an external diameter of 1 mm containing heparin saline was inserted into the left external carotid artery. An initial blood sample of 0.5 ml was withdrawn from the tube and 1 mg FMN dissolved in 0.1 ml water was injected into the artery. Blood samples of 0.5 ml were taken at intervals and substituted by saline. The blood was frozen immediately and stored at -78° until analysis.

2. Male Wistar rats, weighing 250-415 g, were anaesthetized by oral administration of 5 ml 12% (v/v) ethanol per 100 g body weight and the anaesthesia maintained by intraperitoneal injections of 1 ml 20% (v/v) ethanol when necessary. The rat was fixed to heated surgical table and the rectal temperature kept between 37 and 39°. The abdomen was opened and polyethylene catheter was inserted into the bladder for continuous urine collection. The catheterization was made in such a way that the dead space was minimal and the collection made through the polyethylene tube, connected to the catheter. A polyethylene tube was inserted into the left external carotid artery and 1000 u. heparin/kg was injected. A cannula was introduced into the right femoral vein and via a polyethylene tube connected

with a Braun Infusor from which the FMN solution was infused at a rate of 1.32 ml/hour. Blood samples of 50 μ l from the carotid artery and the urine samples were collected before during and after the infusion, at suitable intervals. The operation was done in less than 1 hour and the animals lived for more than 6 hours from the start of infusion. The mean urine production was 1.5 ml/hour decreasing to 0.5 ml/hour during the experiment.

Analytical methods

1. 5 ml 10% trichloroacetic acid was added to the 0.5 ml blood samples and analyses for riboflavin, FMN and FAD were carried out as described by STAHLER (1965).

2. Thin-layer chromatography (pyridine/glacial acetic acid/water: 10/1/40) showed that riboflavin was present as the only flavin in the urine. For analysis, 1.5 ml of 10% trichloroacetic acid was added to 10 μ l urine. After centrifugation, 0.5 ml 1 M K HPO₄ was added to 0.5 ml supernatant and the fluorescence of the mixture was measured as described previously (CHRISTENSEN 1969a). The excretion rate was calculated as μ g/minute.

For the determination of the total flavin concentration the blood samples were transferred directly to 1.5 ml 10% trichloroacetic acid and kept at 37°C for 20 hours or at room temperature for 3 days. After centrifugation, 0.5 ml supernatant was added to 0.5 ml 1 M K HPO₄ and the fluorescence measured as described previously (CHRISTENSEN 1969a). The total flavin concentration in the blood was calculated as μ g/ml.

Results

The distribution of flavins in the blood after the injection of 1 mg FMN is seen in fig. 1. FMN was rapidly dephosphorylated to free riboflavin until a FMN:riboflavin concentration ratio of about 1:3 was reached. The two flavins were then eliminated to the same extent, the concentration ratio remaining unaltered. FAD in the blood was not influenced by the injection during the experimental period. Urine was

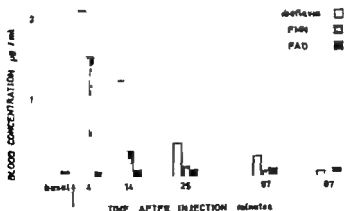


Fig. 1. Blood concentrations of riboflavin, FMN and FAD before and after injection of 1 mg FMN into 300 g rat.

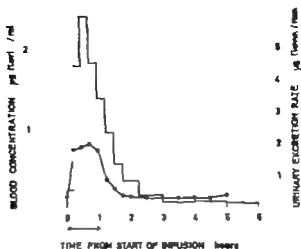


Fig. 2. Total flavin concentration in the blood (—●—) and urinary excretion rate (indicated by columns) of riboflavin following intravenous infusion of 2 mg FMN/kg into rat B. Body weight: 415 g. Infusion rate 1.32 ml/hour and infusion time 1 hour.

collected directly from the bladder two hours after the injection. The urinary recovery was 82% of the dose.

A typical result of experiment 2 is seen in fig. 2. The rat (B) received

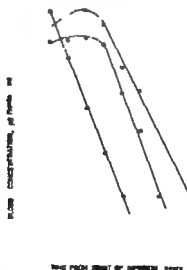


Fig. 3. The relationships between the blood concentration (corrected for basal value) and time after intravenous infusion of FMN into rat A: 250 g (—○—), rat B: 415 g (—●—) and rat C: 385 g (—□—). The ordinate is logarithmic.

Table 1

Half-lives for rate of disappearance from blood and urinary excretion rate, disappearance rate constant and urinary recovery of flavin after intravenous infusion of FMN

Rat no.	Weight g	Half-life, rate of disappearance from blood hr	Apparent first order disappearance rate constant (k_d) hr ⁻¹	Half-life, urinary excretion h	Urinary recovery % of dose
A	250	0.19	3.6	0.45	41
B	415	0.18	3.9	0.40	45
C	385	0.25	2.8	0.45	54
Mean		0.20	3.4	0.43	

2 mg/kg FMN during 1 hour and the curves show the variation in the blood flavin concentration and the urinary excretion rate with time. The blood concentration reached a maximum after $\frac{1}{2}$ hours and decreased slightly after 1 hour although the infusion was continued. After the end of the infusion the blood concentration decreased rapidly and reached the basal value after 3 hours. The urinary excretion rate followed the blood concentration but decreased more slowly and considerable amounts of riboflavin were still excreted after the blood concentration had almost returned to its normal value. Two other experiments (rat A 2.7 mg/kg during $\frac{1}{2}$ hour and rat C 2 mg/kg during 1 hour) gave similar results and fig. 3 shows the blood concentration (corrected for basal value) plotted against time on a semilogarithmic scale. The apparent rectilinear decrease indicates that the elimination includes first order processes at least in the first hour and the average half-life was calculated to be 0.20 hr (12 minutes). Table 1 shows the individual values and the corresponding disappearance rate constants.

Fig. 4 shows the urinary excretion rate, corrected for basal value, plotted against time on a semilogarithmic scale. The urinary excretion rate also decreases exponentially with time and the average half-life is 0.43 hour (26 minutes). Individual values are shown in table 1.

The urinary excretion rate as a function of the blood concentration (both corrected for basal values) is seen in fig. 5. At high blood concentrations the excretion rate increases rectilinearly with the blood concentration indicating excretion by passive glomerular filtration. However the shape of the curve at low blood concentrations suggests that tubular secretion must also be involved. Thus, the curve could be split up in two

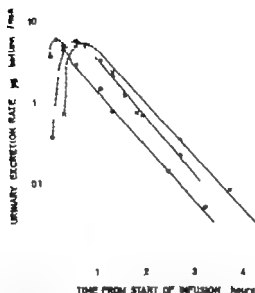


Fig. 4. The relationship between the urinary excretion rate (corrected for basal values) and the time after intravenous infusion of PMN into rat A 250 g (—○—), rat B 415 g (—●—) and rat C 385 g (—x—). The ordinate is logarithmic.

a straight line through the zero point representing excretion by filtration and a curve representing the excretion by tubular secretion which is saturated at a blood level of about 0.2 $\mu\text{g}/\text{ml}$.

Considering the excretion by filtration only the slope of the rectilinear

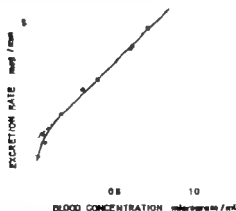


Fig. 5. The relationship between urinary excretion rate (corrected for basal value) and blood concentration (corrected for basal value) of flavin after intravenous infusion of PMN (rat A (—○—) and rat B (—●—)).

part of the curve (4.87 ml/minute) represents a measure of the renal clearance. Assuming a relative blood cell volume of 45 % and that the injected flavin enters the blood cells only to a limited extent (STRIPP 1965), the renal clearance is calculated to be about 0.65 ml/100 g rat/minute (rat B). This is a minimum value as correction for binding to plasma proteins has not been made, but it seems to be of reasonable magnitude as compared with the inulin clearance values of 0.5–1.0 ml/100 g rat/minute found by DICKER (1949). The excretion rate constant (k_{et}) for the excretion by glomerular filtration can also be calculated if the apparent distribution volume (V) is known. If the latter is estimated as the dose infused within the first 15 minutes minus the amount excreted in the same time interval, divided by the blood concentration after 15 minutes the calculations are (rat B)

$$V = \frac{495}{0.61} = 812 \text{ ml} \quad \frac{de}{dt} = k_{et} \cdot V$$

$$k_{et} = \frac{\frac{de}{dt}}{aV} = \frac{b}{V} = 0.37 \text{ hr}^{-1}$$

where $\frac{de}{dt}$ = excretion rate by glomerular filtration

a = blood concentration

b = the slope of the rectilinear part of the curve.

As some flavin is undoubtedly eliminated within the first 15 minutes by routes other than urinary excretion the distribution volume used is presumably too large and the calculated rate constant must therefore be a minimum value.

Discussion

A complete dephosphorylation of FMN in the small intestine of the dog was demonstrated by OKUDA (1958). After oral administration of FMN to man only free riboflavin was increased in the plasma indicating a dephosphorylation process at a site before the vitamin reaches the blood (STRIPP 1965). The present results show that a dephosphorylation occurs rapidly in the blood, so that 4 minutes after the injection more than half of the injected FMN is dephosphorylated. However the dephosphorylation is not complete, and an equilibrium seems to be established at a FMN:riboflavin concentration ratio of about 1:3. As FMN cannot be

detected in the urine it is either not excreted or it is dephosphorylated in the kidneys. As the blood contains both flavins, the disappearance rate constants are possibly not the actual constants for riboflavin, since they are calculated from the total flavin concentration in the blood. If FMN is excreted (and hydrolysed) there should be two constants, one for riboflavin and one for FMN.

The experiments showed that flavin is eliminated very rapidly from the blood, the half-life being only about 12 minutes. The routes of elimination known at present are 1) urinary excretion 2) intestinal secretion and 3) metabolism.

(1) About half of the dose was eliminated by urinary excretion when FMN was infused during $\frac{1}{2}$ -1 hour whereas 82% was excreted when FMN was injected rapidly. If these percentages are compared with the 29 or 7% urinary recovery after intraperitoneal or oral administration in previous experiments (CHRISTENSEN 1969b), it becomes evident that the amount excreted in the urine is great dependent on the rate at which the flavin appears in the blood. The relatively high excretion at low blood levels indicates that tubular secretion must be involved in the excretion by the kidneys. This is interesting, since tubular secretion also seems to play a part in the urinary excretion of riboflavin in man (LEVY & JUSKO 1966 using the data of STRIPP 1965). The suggestion was based on a calculation of the minimal renal clearance and later supported by the finding that probenecid inhibits the excretion of parenterally administered FMN (JUSKO & LEVY 1967).

(2) Secretion of flavin into the intestine after intraperitoneal administration was observed previously (CHRISTENSEN 1969b). Unpublished experiments have shown that after intravenous infusion of FMN riboflavin is secreted not only into the duodenum but into the whole small intestine of the rat. An enterohepatic cycle as assumed to be present in man (LEVY & JUSKO 1967) cannot therefore be the only factor responsible for the intestinal secretion. mucosal secretion must thus also be involved.

(3) Flavin elimination by metabolic processes has been described previously (CHRISTENSEN 1969b). In the present investigation a metabolic elimination is indicated by the observation that the urinary excretion was reduced when flavin entered the blood more slowly. Rapid injections caused a high blood level for a short time whereas gradual administration (infusion) caused a low blood level for a period of time sufficiently long to permit activation of a metabolizing system. However the fact that the blood concentration in all the infusion experiments declined from the maximum value before the infusion stopped points to the elimination by a metabolic system with an increasing capacity so that at the end of the infusion, the elimination rate exceeds the infusion rate.

Summary

- 1 FMN (riboflavin 5 phosphate) injected into the blood stream of the rat was rapidly but not completely dephosphorylated to free riboflavin.
2. Measurements were made of the blood concentration and the urinary excretion rate after intravenous infusion of FMN to rats. Apparent first order functions were found the half lives being about 12 minutes for the rate of disappearance from the blood and about 26 minutes for the urinary excretion rate.
- 3 The data suggest that in the rat riboflavin in addition to glomerular filtration is excreted by tubular secretion.

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Studies on Riboflavin Metabolism in the Rat. IV Riboflavin Elimination after Exclusion of the Portal Circulation

By

Sten Christensen

(Received September 11 1968)

Riboflavin is partially metabolized in the rat after oral, intraperitoneal or intravenous administration (CHRISTENSEN 1969a & b). The site of metabolism is unknown but it seems reasonable to assume that the liver is involved. In the present study the flavin concentration in the blood of rats without portal circulation was measured after the injection of FMN (riboflavin-5 phosphate).

Methods

Principle. The blood circulation of the liver was excluded by ligation of the common hepatic artery and the portal vein. The portal blood was led into a vessel from which it was pumped back into the right femoral vein by means of a peristaltic pump. The method is a simplification of that described by STRIPP & SACHS-HANSEN (1967).

Female Wistar rats weighing 200 to 250 g were anaesthetized with Fluothane ® (halothane) or 50 mg/kg sodium pentobarbital (Nembutal ®) i.p. and placed on a heated surgical table, the rectal temperature being kept between 37 and 39°. Artificial respiration was performed through a tracheal cannula, atmospheric air being blown into the lungs at a frequency of 50 per minute and in a volume of about 5 ml. When fluothane was used as anaesthetic it was administered in a concentration of about 0.3% v/v. The arterial blood pressure was recorded by a membrane transducer connected to a polyethylene tube inserted in the left external carotid artery. The abdomen was opened at the midline and in some cases the renal arteries and veins were ligated. The common hepatic artery was ligated in front of the portal vein between the latter and the bile duct. The portal vein was ligated in the porta hepatis just before the branching and a 1 cm long cannula provided with a soft plastic tube containing heparin saline was introduced into the vein distal to the ligature and fastened by a second ligature just proximal to the superior mesenteric vein. The portal blood was led into an open container holding 10 ml of donor blood from a heparinized donor rat. From the container another tube led to a peristaltic pump which pumped the

blood back into the right femoral vein of the rat. The pumping was started at the same time as the first ligature was fixed round the portal vein. The flow rate into the femoral vein was about 1.5 ml/min and regulated according to the output from the portal vein so that the volume in the container remained constant. Sulphobromophthalein sodium (bromsulphalein \otimes or BSP) was used to control the effectiveness of the procedure. Two mg/kg of FMN and 10 mg/kg of BSP were injected into the left carotid artery after the extrahepatic circulation was established. Duplicate blood samples of 50 μ l were taken for analysis of total flavin and bromsulphalein at suitable intervals. Control rats were treated in the same way except for the extrahepatic circulation. The arterial blood pressure after the operation was 60–90 mm Hg when fluothane was used and 35–50 mm Hg when mebumal-sodium was used as an anaesthetic. There was no difference between the blood pressure in test and control animals. After the experiments, the liver was inspected and in all cases the colour indicated a marked anaemia.

Analytical methods The BSP concentration in the blood was measured as follows. The 50 μ l blood samples were transferred directly into 3.5 ml 0.9% NaCl and the red blood cells were centrifuged immediately. To 3 ml of the supernatant 0.1 ml 1 N NaOH was added, and the extinction measured in a Beckman spectrophotometer model DU at 580 m μ . The blood concentration was calculated as μ g/ml from values obtained with a bromsulphalein standard following subtraction of the basal blood values.

For total flavin analysis the 50 μ l blood samples were transferred into 1.5 ml 10% trichloroacetic acid and the total flavin concentration in the blood was determined as previously described (CHRISTENSEN 1968b).

Results

Fig. 1 shows the results in two rats on fluothane anaesthesia with porta-cava shunt and intact kidneys. After 45 minutes, BSP had completely disappeared from the blood in the control rats whereas a high concentration remained in the blood of the test rats. Though the blood concentration did not become constant during the experiment, the considerable difference between the blood concentrations in rats with and without porta-cava shunt indicates a complete or almost complete exclusion of the liver circulation. Furthermore, a large difference in flavin concentration was found between the test and control animals. Fifteen minutes after the injection, the increased amount of flavin in the blood (i.e. minus the initial blood flavin) was 7–9 times as large as in the control animals and after 45 minutes 30–40 times as large as in the control animals. The flavin concentration did not become constant during the experiment but there is only a small difference between the 30 and 45 minutes values for rat B. The test animals lived for about 1 hour after the injection.

Similar results were obtained in a fluothane anaesthetized rat with porta-cava shunt and ligated kidneys (fig. 2). The liver exclusion is indicated by the bromsulphalein curves and since the urinary excretion was prevented the blood flavin concentration in the control rat decreased more slowly and did not reach the initial value. The rat (C) lived for 1 hour following the injection.

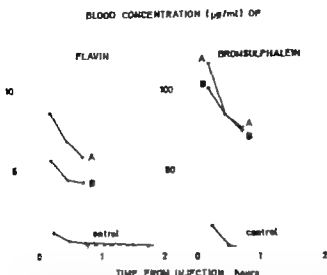


Fig. 1 The blood concentration of total flavin and BSP (sulphobromophthalein sodium) in rats with porta-cava shunt after intra-arterial injection of FMN (2 mg/kg) and BSP (10 mg/kg). The control rat had no porta-cava shunt. Anaesthesia: Floothane. Body weight: Rat A 230 g, rat B 230 g, and control rat 225 g.

Two rats on mebumal-sodium anaesthesia with porta-cava shunt and ligated kidneys were kept alive sufficiently long to obtain constant blood levels of both bromsulphalein and flavin (fig. 3). The increased amounts of flavin in the blood of the test rats were 2–3.4 and 7–8 times as high as those in the control rats after 15, 45 and 90 minutes, respectively.

Table 1

Increased flavin in the blood (i.e. minus the basal amount) 15, 45 and 90 minutes after injection of 2 mg/kg FMN (riboflavin-5-phosphate).

Operation (anaesthesia)	Rat no.	Increased blood concentration (µg/ml) after		
		15 min	45 min	90 min
Liver excision (floothane)	A	8.31	5.61	
	B	3.34	4.01	
	control	0.79	0.14	0.02
Liver excision Kidney excision (floothane)	C	7.04	4.77	
	control	2.24	0.99	0.32
Liver excision Kidney excision (mebumal Na)	D	5.92	4.70	3.70
	E	4.78	3.74	3.40
	control	2.22	0.88	0.15

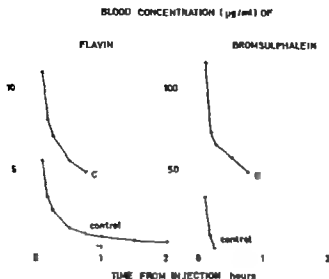


Fig. 2. The blood concentration of total flavin and BSP in a rat with porta-cava shunt and ligated renal vessels after intra-arterial injection of FMN (2 mg/kg) and BSP (10 mg/kg). The control rat had no porta-cava shunt. Anaesthesia: Fluothane. Body weight: Rat C 230 g and control rat 215 g.

As seen in fig. 2 and 3 the flavin concentrations in the control rats with ligated kidneys did not reach the basal flavin concentration as seen in the control rat with intact kidneys, but seemed to approach asymptotically

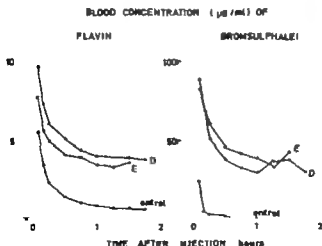


Fig. 3. The blood concentration of total flavin and BSP in rats with porta-cava shunt and ligated renal vessels after intra-arterial injection of FMN (2 mg/kg) and BSP (10 mg/kg). The control rat had no porta-cava shunt. Anaesthesia: Methuhalohydium. Body weight: Rat D 229 g, rat E 225 g and control rat 230 g.

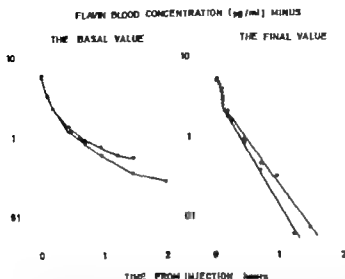


Fig. 4 The blood concentration of total flavin minus the basal value (left curves) and total flavin minus the final value (right curves) after intra-arterial injection of FMN (5 mg/kg) in rats with ligated renal vessels. The ordinate is logarithmic. (—●—) control rat from fig. 2 and (—○—) control rat from fig. 1.

a level which is 2-3 times higher. If the increased blood concentrations are plotted directly against time on a semilogarithmic scale (fig. 4 left curve), the decrease is seen to be non-exponential. However if the values which the blood concentrations seem to approach (i.e. the final values) are subtracted, straight lines appear indicating that the elimination to this blood level is exponential. The apparent first order disappearance rate constants were derived from the slopes and found to be 1.12 and 1.40 hr⁻¹ respectively.

Discussion

The disappearance of BSP from the blood shows that the liver is completely or almost completely excluded from the blood circulation by the present method. The considerable decrease in the blood concentration of flavin and BSP during the first hour after the injection is presumably due to delayed distribution caused by the extrahepatic circulatory system. As the decrease of the two compounds seems to be parallel, intestinal secretion cannot play any great part in the elimination of flavin from the blood.

When comparing the results it must be taken into account that the test rats have 10 ml more blood in their circulatory system than the control

rats. This means that the measured blood flavin concentrations in the test rats are too small and consequently that the difference between test and control concentrations should be higher than shown above i.e. even more significant.

The experiments show that the blood flavin concentration after injection of FMN is much higher when the liver is excluded from the circulation. When the kidney circulation is interrupted the difference between the blood concentrations in the test and control rats must be due to elimination of flavin in the liver. Such an elimination may occur in two different ways i.e. by metabolism and by excretion in the bile.

Biliary excretion of riboflavin has been shown in the dog by TEDSCH (1954) but not directly in the rat. YAGI *et al* (1966) found an accumulation of riboflavin in the small intestine after subcutaneous injection of ^{14}C -riboflavin and suggested the presence of an enterohepatic cycle. However, intestinal secretion could also be involved. Considering the molecular weight (376) and the molecular structure, biliary excretion is probable but nothing is known about its quantitative importance. Nonetheless, it seems likely that elimination from the liver which is expressed by the difference in blood concentration between control and test animals, is mainly due to the metabolism of riboflavin, although the nature of the metabolizing process is unknown.

The disappearance of flavin from the blood in rats without kidney circulation indicates an extrarenal elimination. The curves (controls in fig. 2 and 3) indicate that not all of the excess flavin is eliminated from the blood when the kidneys are ligated, whereas a normal blood concentration is reached within 2 hours when the kidney circulation is intact (fig. 1 control). This points to the existence of a lower threshold concentration in the blood for the extrarenal elimination. The elimination to this threshold concentration seems to be exponential (fig. 4) and the calculated disappearance rate constant (mean 1.26 hr^{-1}) should be equal to the sum of the rate constants for the elimination processes other than urinary excretion i.e. metabolism, intestinal secretion and biliary secretion. This compares reasonably well with the previously found disappearance rate constant, 3.43 hr^{-1} which includes urinary excretion (CHRISTENSEN 1968b).

The present and previous papers in this study deal with only one aspect of the biological fate of flavin, i.e. the disappearance of the vitamin in a form which can be detected by the fluorometric method. The question of the metabolites and their fate is still unsolved and will be treated in further studies with ^{14}C riboflavin.

Summary

By porta-cava anastomosis and ligation of the common hepatic artery in the rat, the liver was excluded from the circulatory system. After the injection of FMN the flavin concentration in the blood was much higher than that in control rats with normal hepatic circulation. From the results it is concluded that most, if not all of the metabolic destruction of flavin takes place in the liver.

Acknowledgements

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Failure to Produce Heparin Osteoporosis in Rats

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Long term administration of subcutaneously injected water soluble heparin has been reported to cause osteoporosis with vertebral compression fractures in man (GRIFFITH *et al.* 1965 JAFFE & WILLIS 1965 MÜLLER & WOLFF 1966). Similarly experiments with heparin administration to rats have indicated an effect on the skeleton resulting in osteoporosis (ASHER & NICHOLS 1965). Moreover in *in vitro* experiments with cultures of bone, resorption increased in the presence of heparin and suboptimal concentration of parathyroid hormone (GOLDHABER 1965).

In none of the studies mentioned above the diagnosis of osteoporosis was based on quantitative measurements of mineral or bone mass. The objective of the present study was to measure quantitatively the possible effect of heparin on the mineral mass in the skeleton of rats.

Material and Methods

Forty female, albino rats (weight range 70-240 g) were arranged in weight-matched pairs. One rat in each pair was given daily subcutaneous injection of 1 l.u. per g body weight of water-soluble sodium-heparin¹⁾ the other rat received the same volume of physiological N Cl solution. The schedule of daily injections was maintained for 100 days. Twenty-four hours after the last heparin injection the rats were killed.

The animals were weighed once more and the hind limbs were exarticulated in the hip joints. One femur was carefully cleaned of soft tissue to allow evaluation of volume, the remaining parts of the hind limbs were used for histological examination.

Immediately after dissection the femora were submerged in water later the volume was determined in the following way

The bones were suspended on a fine copper wire and weighed in air and in water to an

¹⁾ Heparin ®, Vitrum, Stockholm, Sweden.

accuracy of 0.1 mg. The water was kept at -4° and a small amount of detergent was added to decrease the effect of surface tension. From the weight in air and the weight in water volume and density could be calculated.

After weighing, the bones were ashed for 24 hours at 550° to white ash which was weighed.

The carcass minus the two removed hind limbs was dried and ashed for 4 hours at 700° and the ash was weighed to an accuracy of 0.1 mg.

One femur from each animal was decalcified, embedded in paraffin, sectioned and stained with hematoxylin-eosin and according to van Gieson.

Results

There was no difference in the final weight between heparin-treated and control animals and therefore, no indication of effects of heparin on the growth rate (table 1).

There were no differences in volume, ash content, density or the ash content per volume bone (spec. ash weight) between the femora of heparin treated and control rats, nor was there any difference between the total ash contents of the animals (table 1).

The relationship between spec. ash weight and density of the femora was also the same in heparin-treated and control animals (figure 1).

The range of body weight at the beginning of the experiment was chosen in order to study a possible effect, of growth rate on heparin osteoporosis. There was no such influence of primary body weight on density or ash content per volume of bone between the two groups.

The histological examination revealed no difference between the two groups.

Table 1

Variables of bone mass in heparin-treated and control rats.
Averages and probability levels of difference λ .

	Heparin	Control	P)
Body weight at death (g)	201	200	$0.7 > P > 0.6$
Ash content, carcass minus hind limbs (g)	6.20	6.03	$0.4 > P > 0.3$
V lume, femur (ml)	0.397	0.398	$0.3 > P > 0.2$
Density femur	1.49	1.49	$0.7 > P > 0.6$
Spec. ash weight, femur (g/ml)	0.556	0.561	$0.8 > P > 0.7$

) T-test of difference between paired observations.

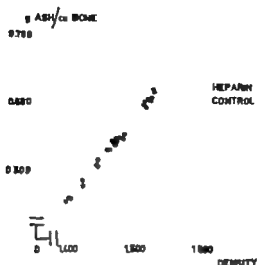


Fig. 1 The relationship between spec. ash weight and density in heparin treated and control rats. There is no significant or suggestive difference between the groups.

Discussion

The dose administered in the present study compares with the highest recommended human dose and is more than three times the doses reported to produce osteoporosis in man. In a preliminary study this dose had been demonstrated to at least quadruple the coagulation time at 7 and 11 hours after the injection. 24 hours after the injection the coagulation time was still significantly increased. The time period of heparin administration, 32 days, should have produced a measurable change in the mineral mass considering the high rate of bone mineral turn-over in the rat (BAUER *et al* 1955), had such an effect been present.

It must therefore be concluded that heparin does not produce osteoporosis in rats.

Summary

One i.u. per gram body weight of water-soluble heparin was given to rats for 32 days. There was no effect on the bone mineral mass.

Acknowledgement

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The Effect of Protriptyline on Dopamine and Noradrenaline Metabolism in Normal and Atrophied Rat Salivary Glands In Vitro

By

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The uptake, storage and metabolism of catecholamines in sympathetic nerves have been extensively studied during the last decade (for review see e.g. IVERSEN 1967). Recently it has been shown that in rabbit brain cortex slices, dopamine (DA) is primarily converted to the phenolic acids 3,4-dihydroxyphenylacetic acid and homovanillic acid, whereas the major metabolic products of noradrenaline (NA) are the phenolic glycols 3,4-dihydroxyphenylglycol and 3-methoxy-4-hydroxyphenylglycol (RUTLEDGE & JONASON 1967). The metabolism of these catecholamines has also been investigated in rat salivary gland slices (JONASON unpublished). The metabolic pathways of DA were found to be the same as in the brain cortex whereas those of NA were different. The major metabolite of exogenously added NA in this tissue was found to be normetanephrine (NM) while newly synthesized NA from DA was primarily deaminated to the corresponding phenolic glycols. It is also known that the salivary glands have a particularly high activity of the enzyme catechol-O-methyl transferase (COMT) (AXELROD, ALBERS & CLEMENTI 1959). After ligation of the excretory ducts of rat salivary glands, the gland cells atrophy with a concomitant decrease in weight (JUNQUEIRA 1951, BHASKAR, BOLDEN & WEINMANN 1956, STANDISH & SHAFER 1957). The adrenergic nerve terminals do not show a corresponding atrophy. The NA content per gland is almost unchanged and the network of the NA terminals is much denser than in normal glands (ANDÉN, NORBERG & OLSON 1966). However, the monoamine oxidase (MAO) activity is reduced by more than 60 per cent by this procedure (ALMGREN *et al.* 1966) and a severe loss of COMT activity is found when atrophied salivary glands are incubated

with DA and NA (JONASON unpublished). About one third of the MAO activity is localized inside the adrenergic nerves whereas about two thirds of the enzyme activity is found extraneuronally (ALMGREN *et al* 1966). COMT is to a very large extent localized to the parenchymal cells in rat salivary glands (JONASON unpublished).

An important metabolic function of the adrenergic neuron is its ability to concentrate exogenously administered catecholamines (DENGLER, SPIEGEL & TITUS 1961a & b; ROSS & RENYI 1964; HAMBERGER & MASUOKA 1965). A concentrating mechanism is localized at the level of the cell membrane of the peripheral adrenergic nerves (HILLARP & MALMFORS 1964) as well as in brain neurons (HAMBERGER & MASUOKA 1965). Protriptyline (PTP) is one of the most potent inhibitors of this mechanism in peripheral tissues (CARLSSON & WALDECK 1965) and brain (CARLSSON *et al* 1966). The effect of this agent on the metabolism of DA and NA in rabbit brain cortical slices and slices of rabbit caudate nucleus has recently been investigated (JONASON & RUTLEDGE 1968a & b). It was found that PTP had no effect on the metabolism of DA and NA in the DA neurons in the caudate nucleus. However in cortex cerebri (containing primarily NA neurons) a greater reduction of the acid and neutral catabolites of NA as opposed to DA was seen after PTP treatment. A possible explanation for this phenomenon is that NA utilizes the "membrane pump" mechanism to a greater extent than DA. However BURGEN & IVERSEN (1965) in experiments with isolated hearts found that DA has a higher relative affinity for the uptake sites than NA. The present study was performed to investigate the action of PTP on the metabolism of DA and NA in normal and atrophied rat salivary gland slices, in order to elucidate the relative importance of the intraneuronal and extraneuronal metabolism of these amines.

Material and Methods

Adult male Sprague-Dawley rats weighing about 200 g were used. Under pentobarbital sodium anesthesia (40 mg/kg i.p.) the secretory ducts of the left submaxillary and sublingual glands were ligated near the hilum. Fourteen days after the operation, when the left glands were maximally atrophied, the animals were sacrificed by being bled out under light chloroform anesthesia or by a blow on the head. About half of the animals were pretreated with 10 mg/kg of PTP p.o. 30 min. before being killed. The submaxillary as well as the sublingual glands on each side were removed, weighed and immediately sliced. In general, about five slices were obtained from the atrophied glands and about ten slices from the normal glands. The slices (all of the slices obtained from one gland inserted into a single incubation flask) were pre-incubated in 5 ml of Krebs-Henseleit solution for 10 min. at 37°C in 95 per cent O₂ - 5 per cent CO₂ atmosphere (RUTLEDGE & WERNER 1967). Then 5.670×10^{-1} moles of either C¹⁴-DA (46.5×10^{-9} curies) or H³-NA (6.66×10^{-7} curies) were added and the flasks incubated for an additional 20 min. Control samples were pre-

pared by addition of 2 ml 2 N-HCl to the flasks before the pre-incubation period. All incubations were terminated by the addition of 2 ml of 2 N-HCl.

When the retention of the amines was measured, the slices were blotted and dried on filter paper after the 20 min. incubation and incubated for an additional 10 min. in 5 ml of fresh substrate-free Krebs-Henseleit solution. The slices were then blotted and dried again and inserted into a tube containing 2 ml 2 N-HCl. After homogenization, centrifugation and filtration the extracts were freeze-dried and the total radioactivity retained by the slices was determined in a Packard Tricarb scintillation apparatus.

The procedures used in this study have been described in detail in previous publications (RUTLEDGE & JONASON 1967 JONASON & RUTLEDGE 1968a) and thus will be presented here only in general terms. DA and NA were isolated by desorption on to a Dowex 50 column, from which these amines were eluted into separate fractions. When methoxytyramine (MTA) and normetanephrine (NM) were determined the neutralized extracts were first passed over alumina, where the two catecholamines, DA and NA, were adsorbed. The alumina effluent containing MTA and NM was then passed over Dowex 50 column and the two amines were eluted in separate fractions. The amine eluates were freeze-dried and the radioactivity was measured by liquid scintillation technique. Recoveries of the amines were determined in each sample by adding carrier substances immediately on termination of the incubation and quantitating the amount remaining in the eluate after the separation procedures. The amount of amines recovered was quantitated by the fluorescence emitted at 335 nm on activation at 280 nm. When the total of the deaminated catabolites was determined, the extract was passed over Dowex and the catabolites in the effluent were extracted with ether. The ether volume was reduced to dryness, scintillation cocktail fluid was added and the radioactivity was determined in a Packard Tricarb scintillation apparatus.

The values are expressed as means \pm the standard errors of the mean (S.E.M.) and are presented in terms of moles $\times 10^{-12}$ per flask obtained after 20 min. incubation period. The values of the amines were calculated by correcting for efficiency recovery aliquot factors and the specific activity of the precursor. The values of the combined acid and neutral catabolites as well as the values for the retention of the amines were calculated in the same manner except that no recovery values could be assessed, thus no correction for this variable was made. It was assumed that the recoveries of these two groups of compounds were relatively constant. All values except the retention values represent amines + catabolites in the media as well as that in the tissue. Where nothing else is mentioned the P-values have been calculated by Student's t-test.

Results

The effect of PTP on the metabolism of DA

The results are presented in table 1. The metabolic rate of DA is high in normal salivary glands. After 20 min. incubation only 32 per cent of the substrate is not metabolized (compared to the control values). Normally large amounts of deaminated catabolites are formed and it is known that the major catabolites of this amine are the phenolic acids 3,4-dihydroxyphenylacetic acid and homovanillic acid (RUTLEDGE & JONASON 1967 JONASON unpublished). MTA and NA are formed from DA in this preparation whereas NM is found only in negligible quantities. As seen

Table 1

The effect of protriptyline on the metabolism of dopamine in normal and atrophied rat salivary glands.

Treatment		DA	NA	MTA	NM	Acid and neutral catabolites
NORMAL GLANDS	Mean	1615	201 (86)	193 (175)	19.9 (0.5)	1107 (1083)
	S.E.M.	157	17.2	15.0	~7	140
	n	13	10	9	9	11
ATROPHIED GLANDS	Mean	3148	264 (149)	42.2 (24.6)	20.9 (1.5)	826 (802)
	S.E.M.	236	21.3	5.4	3.4	93.8
	n	8	11	7	7	7
NORMAL GLANDS + PTP 10 mg/kg	Mean	1267	63.6 ^a (0)	388 (370)	1.1 (1.7)	723 (699)
	S.E.M.	108	3.7	14.4	4.4	61.2
	n	14	12	6	6	12
ATROPHIED GLANDS + PTP 10 mg/kg	Mean	3088	151 (36)	135 (117)	20.0 (0.6)	248 (224)
	S.E.M.	708	12.4	5.4	4.5	21.9
	n	13	13	6	6	13
CONTROL	Mean	5052	113	17.6	19.4	24.4
	S.E.M.	469	31.8	2.9	1.6	9.3
	n	7	7	6	6	8

Slides of normal and atrophied rat salivary glands were incubated for 20 min. with 5.670×10^{-12} moles of C^{14} -dopamine (DA) (46.5×10^{-6} curies). Some animals were pretreated with protriptyline (PTP) 10 mg/kg i.p. 30 min. before the animals were killed. Control corresponds to samples in which 2 ml of 2 N-HCl were added to the incubation fluid before incubation. Recovery of DA equals 86.0% \pm 1.4 noradrenaline (NA) 93.9% \pm 1.1 methoxytyramine (MTA) 73.2% \pm 1.8, and normetanephrine (NM) 82.3% \pm 0.8. The values of DA, NA, MTA and NM but not the values of acid and neutral catabolites were corrected for recovery. ^a represents the number of experiments. S.E.M. represents the standard error of the mean. Values in brackets represent values in which the control values have been subtracted. Values are presented as moles $\times 10^{-12}$ and represent amine or catabolites in the media as well as that in the tissue. ^b Significantly different from normal values: (P < 0.05) ^c P < 0.01 ^d P < 0.001.

from table 1 atrophy increased the amount of nonmetabolized DA (P < 0.001) and possibly also the level of NA (P < 0.05) whereas the level of MTA was markedly decreased (P < 0.001). Recently it has been shown that atrophy of the salivary glands produces a severe loss of

COMT activity whereas sympathetic denervation of the tissue increases the levels of the 3-O-methylated products (JONASON unpublished). Thus the major part of the enzyme COMT is localized to the parenchymal cells of the salivary glands. The amount of acid and neutral catabolites might be somewhat lowered ($P < 0.1$) by atrophy.

In normal glands pretreatment of the animals with PTP reduced the level of NA to zero ($P < 0.001$) and that of acid and neutral catabolites to 64.5 per cent of the normal ($P < 0.05$) – calculated after subtracting the control values. By this procedure the amount of MTA increased by more than twice ($P < 0.001$). In the atrophied glands PTP produced a pronounced decrease of acid and neutral catabolites (21 per cent of the normal – $P < 0.001$) the amounts found being significantly lower than in both atrophied and PTP pretreated normal glands ($P < 0.001$). The level of MTA in these PTP treated atrophied glands was actually increased as compared to the atrophied ones ($P < 0.001$) but decreased as compared to the normal ($P < 0.01$) and the PTP pretreated normal glands ($P < 0.001$). NA which was not formed in the normal glands after PTP was found in small quantities in the PTP treated atrophied ones ($P < 0.001$). The amount of nonmetabolized DA was found to be about the same in atrophied glands with and without treatment with this agent.

It is rather surprising that the value of C^{14} -NA in the PTP-treated normal glands is less than zero after subtraction of the control value. However this phenomenon can be explained as follows. The relatively large control values of C^{14} -NA (which have been subtracted from the experimental value) are probably due to partial contamination with the radioactive precursor (about 2 per cent of DA in the control) during the separation procedures. In contrast to the control 1267×10^{-12} moles of DA is found after 20 min. incubation in the PTP treated normal glands. Two per cent of this value yields about 25×10^{-12} moles as the actual control values of C^{14} -NA in this case. If this value is subtracted from the experimental value it appears that some small amounts of NA might be synthesized in normal glands after pretreatment with PTP.

The effect of PTP upon the metabolism of NA

Normally the metabolic rate of NA was slower than that of DA in the salivary glands as seen in table 2. About 56 per cent of the substrate was nonmetabolized after 20 min. incubation (as compared with the control values). Large amounts of the 3-O-methylated product NM was found but only relatively small amounts of acid and neutral catabolites were detected. This is partly a reflection of the fact that the major decamina-

Table 2

The effect of protriptyline on the metabolism of noradrenaline in normal and atrophied rat salivary glands.

Treatment		NA	NM	Acid and neutral catabolites
NORMAL GLANDS	Mean	2486	530	246
			(513)	(238)
	S.E.M	103	30.4	17.2
	n	8	10	8
ATROPHIED GLANDS	Mean	4023	876	218
			(706)	(210)
	S.E.M	161	15.8	17.2
	n	6	10	6
NORMAL GLANDS + PTP 10 mg/kg	Mean	2206	555	186
			(538)	(178)
	S.E.M	139	26.9	7.3
	n	11	6	12
ATROPHIED GLANDS + PTP 10 mg/kg	Mean	3627	141	42.3
			(124)	(34.8)
	S.E.M	231	9.0	2.3
	n	14	3	14
CONTROL	Mean	4445	17.0	7.5
	S.E.M	392	4.1	0.3
	n	6	3	7

Effect of normal and atrophied rat salivary glands were incubated for 20 min. with 5670×10^{-12} moles of H^3 -noradrenaline (NA) (6.66×10^{-12} curies). Some animals were pretreated with protriptyline (PTP) 10 mg/kg i.p. 30 min. before the animals were killed. Control corresponds to samples in which 2 ml of 2 N-HCl were added to the incubation fluid before the incubation. Recovery of NA equals $93.9\% \pm 1.1$ and that of normetanephrine (NM) $82.3\% \pm 0.8$. The values of NA and NM but not the values of acid and neutral catabolites were corrected for recovery. Values in brackets represents the number of experiments. S.E.M. represents the standard error or the mean. Values in brackets represent values in which the control values have been subtracted. Values are presented as moles $\times 10^{-12}$ and represent amount or catabolite in the media as well as that in the tissue.) Significantly different from normal values at $P < 0.01$) $P < 0.001$

tion products of NA are the phenolic glycols (RUTLEDGE & JONASON 1967 JONASON unpublished). The glycols are not extracted into ether as well as the major catabolites of DA, which are phenolic acids. However DA is metabolized at a higher rate than NA because of its higher affinity for extraneuronal and intraneuronal MAO (JONASON unpublished). Atrophy

Table 3

The effect of protriptyline on the retention of dopamine and noradrenaline by slices of atrophied and normal salivary glands.

		DA	NA
NORMAL GLANDS	Mean	349 (338)	347 (342)
	S.E.M.	39.6	39.4
	n	12	8
ATROPHIED GLANDS	Mean	361 (350)	313 (308)
	S.E.M.	42.9	23.3
	n	8	7
NORMAL GLANDS + PTP 10 mg/kg	Mean	112 (101)	65.0 (60.3)
	S.E.M.	8.6	3.7
	n	5	6
ATROPHIED GLANDS + PTP 10 mg/kg	Mean	119 (108)	210 (205)
	S.E.M.	4.7	17.4
	n	5	6
CONTROL	Mean	10.8	4.7
	S.E.M.	2.3	1.3
	n	3	2

Slices from normal and atrophied rat salivary glands were incubated for 20 min. with 5670×10^{-12} moles of either C^{14} -dopamine (DA 46.5×10^{-9} cpm) or H^3 -noradrenaline (NA 6.66×10^{-7} cpm). The values represent the amount of amine (or catabolite) retained by the slices after a 10 min. post-incubation in substrate-free medium and are expressed as moles of amine $\times 10^{-12}$.) Significantly different from normal values at $P < 0.05$) $P < 0.01$) $P < 0.001$

produced a severe reduction of NM (14 per cent of normal) and an increase in the amounts of nonmetabolized NA (91 per cent of the control value), whereas the level of acid and neutral catabolites was not significantly affected. The results are in agreement with the data of a previous study on the metabolism of DA and NA in normal atrophied and sympathetically denervated salivary glands (JONASON unpublished). PTP pretreatment of normal glands reduced the level of acid and neutral catabolites to 75 per cent of the normal (after subtracting the control values) ($P < 0.01$), whereas the levels of NM and NA were almost unchanged. When atrophied glands were treated with the same agent the

levels of acid and neutral catabolites were reduced to 15 per cent of the normal ($P < 0.001$) the values being significantly lowered even as compared to that of atrophied and PTP pretreated normal glands ($P < 0.001$). NM was markedly lowered after this treatment but significantly higher than in the glands after atrophy only ($P < 0.05$). The amount of non metabolized NA in the atrophied glands was not significantly affected by PTP treatment.

The effect of PTP on the retention of DA and NA

As seen from table 3 approximately equal amounts of DA and NA were normally retained by the slices. This retention ability was not affected by atrophy. PTP pretreatment of normal glands reduced the levels of retained DA and NA to 30 and 18 per cent respectively of the normal. Obviously PTP has a greater effect on the retention of NA than that of DA ($P < 0.025$ - analyzed by an elementary analysis of variance). When atrophied salivary glands were pretreated with this agent the amount of DA retained was lowered to the same extent as in PTP-treated normal glands. In contrast NA in PTP treated atrophied glands was reduced but was significantly higher than the level in PTP treated normal glands ($P < 0.001$ - analyzed by an elementary analysis of variance). In PTP treated atrophied glands the amount of NA retained was significantly higher than that of DA ($P < 0.001$ - analyzed by an elementary analysis of variance).

Discussion

In a previous investigation the metabolism of DA and NA in slices of normal, atrophied and postganglionically sympathectomized rat salivary glands (JONASON unpublished) was investigated. The present study was mainly concerned with the effects of PTP on the metabolism of these catecholamines in normal and atrophied salivary glands. This agent is known to be one of the most potent inhibitors of the amine concentrating mechanism at the level of the cell membrane of the adrenergic nerves (CARLSSON & WALDECK 1965). LUNDBORG & STITZEL (1967) have presented evidence from subcellular distribution studies in the mouse heart, that the uptake of the amines is inhibited at the cell membrane level rather than at an intraneuronal storage site. It is obvious from this investigation that PTP prevents the deamination reactions of both DA and NA in the normal glands, the amounts of acid and neutral catabolites being slightly reduced in both cases. However in the PTP treated normal glands the catecholamines can still be deaminated by extraneuronal MAO which

constitutes about two thirds of the activity of this enzyme in the salivary glands (ALMGREN *et al* 1966). This situation might explain why the deamination reactions are not greatly decreased by this treatment. Atrophy abolishes the activity of extraneuronal MAO (ALMGREN *et al* 1966). After this, PTP exerts a much more marked effect on the levels of the deaminated catabolites. Only 18 per cent of the normal values of acid and neutral catabolites are formed from NA. The corresponding value of DA is 21 per cent. It is to be noted that this marked decrease in the deamination reactions by PTP treatment of atrophied glands is accompanied by a severe loss of activity of both COMT and MAO (JONASON unpublished). The substrate concentration for the "membrane pump" is probably increased by atrophy but the uptake by the cell membrane is inhibited by PTP.

As shown previously (JONASON unpublished) atrophy produces a severe loss of COMT activity. The amounts of MTA and NM formed after incubation with DA and NA, respectively are markedly reduced by this procedure (about 14 per cent of the normal). This finding indicates that COMT is to the greatest extent localized to the parenchymal cells in the salivary glands. Pretreatment of the animals with PTP increases the amount of MTA formed from DA in normal glands by more than twice, whereas the level of NM from NA (table 2) is almost unchanged. This is in agreement with findings from rabbit brain cortex *in vitro* (JONASON & RUTLEDGE 1968a) and *in vivo* studies (CARLSSON *et al* 1966, GLOWINSKI *et al* 1966). Sympathetic denervation of the glands appears to have a somewhat larger effect on the levels of the methoxylated amines (JONASON unpublished) than treatment with PTP. Sympathectomy increases the level of MTA by about 3 times and that of NM to 172 per cent. A reason for this discrepancy might be that some part of the substrate can be taken up by the adrenergic nerves after PTP treatment by competition between the drugs for the uptake sites. The level of MTA formed from DA and NM from NA in the PTP treated atrophied glands is markedly increased as compared to the atrophied glands but decreased as compared to normal glands. This indicates that there is a small amount of COMT activity present extraneuronally after atrophy. Treatment with this "membrane pump" blocking agent increases the substrate concentration for extraneuronally localized COMT resulting in an increase in the levels of the methoxylated amines. The amount of NA formed from DA is reduced to zero in PTP-treated normal glands. However in the PTP treated atrophied glands synthesis of NA actually occurs, further supporting the contention that some part of DA can be taken up by the adrenergic nerves after PTP. Atrophy which markedly reduces the activity of both COMT and extraneuronal MAO increases the substrate concentrations not only for the

small amounts of COMT and MAO which still remain but also for the amine uptake mechanism at the nerve cell membrane. Thus, small amounts of DA can be taken up possibly by competition with PTP and further converted to NA.

Normally approximately equal amounts of DA and NA are retained by the slices. Atrophy does not significantly affect this retention ability of the slices. This phenomenon could be interpreted as follows. Normally the amine uptake mechanism might be saturated. In this case atrophy which results in higher amine concentrations, would not increase the retention ability of the slices. Furthermore, loss of possible extraneuronal binding sites in the parenchymal cells (ALMGREN ANDÉN & WALDECK 1965) does not seem to affect the retention ability of catecholamines by the salivary gland slices. PTP reduces the retention of DA and NA in normal glands to 30 and 18 per cent, respectively. Thus, PTP exerts a greater effect on the retention of NA than that of DA ($P < 0.025$ - analyzed by an elementary analysis of variance). JONASON (unpublished) found that sympathectomy reduced the retention ability of salivary gland slices to about 40 per cent. Probably there are some extraneuronal binding sites for amines in the salivary gland. After PTP-treatment the values of retained DA are reduced to about the same degree as after sympathectomy whereas the values of retained NA after PTP seem to be somewhat lower. Consequently PTP appears to have some extraneuronal effect on NA metabolism. In the atrophied glands, PTP reduced the retention ability of DA to about the same extent as in normal glands, whereas that of NA was reduced to only 60 per cent of the normal. Obviously the retention ability of NA in these PTP treated atrophied glands is reduced to a smaller extent than in PTP treated normal ones. ($P < 0.001$ - analyzed by an elementary analysis of variance). The level of retained NA in these PTP treated atrophied glands is markedly elevated as compared to the level of retained NA after sympathectomy (JONASON unpublished). These data suggest that NA has a greater affinity for the PTP sensitive uptake mechanism. As mentioned previously atrophy markedly reduces both COMT and extraneuronal MAO activity (ALMGREN *et al* 1966, JONASON unpublished). The substrate concentration for the uptake mechanism is consequently increased by this procedure. The NA, which has a greater affinity for this "membrane pump" may be taken up into the nerves by competition with PTP to a greater extent than DA, which has less affinity for this mechanism. The same phenomenon has been observed in PTP treated rabbit brain cortex slices (JONASON & RUTLEDGE 1968a). If the DA concentration was decreased ten-fold these investigators found that PTP exerted a more marked effect on the uptake of this amine into the nerves. JONASON & RUTLEDGE (1968a) also found that PTP reduced the

catabolism of NA to a greater extent than that of DA. In the salivary glands, it has not been possible to demonstrate such a difference. BURGEN & IVERSEN (1965) in experiments on isolated hearts have found that the relative affinities of DA and NA for the uptake mechanism of the cell membrane is 1:6:1. These figures have been determined by measuring the inhibition of uptake of labelled noradrenaline by the administration of unlabelled agents. However the different effects of these drugs on e.g. vascular flow might interfere with the results obtained. The data in this study support the view that NA has a greater affinity than DA for the PTP-sensitive uptake mechanism of the adrenergic neurons.

Summary

Slices of normal and atrophied rat salivary glands were incubated with labelled dopamine or noradrenaline. It was seen that the metabolic rate of dopamine was higher than that of noradrenaline. Atrophy markedly reduced the amount of methoxytyramine and normetanephrine, when dopamine and noradrenaline were used as substrates. These data indicate that the major part of the enzyme catechol-O-methyl transferase is localized to the parenchymal cells. The levels of acid and neutral catabolites was not affected so much by atrophy indicating a large intraneuronal capacity of deamination. When dopamine was used as substrate it was seen that in the normal glands protriptyline pretreatment of the animals produced an increase in methoxytyramine, a decrease in the levels of acid and neutral catabolites (65 per cent of normal) and a reduction of the amount of noradrenaline to zero. In the atrophied glands, the same pretreatment resulted in a marked decrease of acid and neutral catabolites (21 per cent of normal) whereas the level of methoxytyramine was increased. The data show the relative importance of the "membrane pump mechanism" and the intraneuronal part of monoamine oxidase (which is known to be responsible of about one third of the total monoamine oxidase activity in the whole gland) and further support the view of the extraneuronal localization of catechol-O-methyl transferase.

When noradrenaline was used as substrate much smaller amounts of deaminated catabolites were isolated as compared to when dopamine was used as substrate. The quantitatively predominant metabolite was normetanephrine. Protriptyline pretreatment reduced the level of acid and neutral catabolites in the normal glands whereas in the atrophied glands the levels of the deaminated catabolites after the same pretreatment were markedly reduced. Atrophy alone did not affect the amount of deaminated catabolites of noradrenaline. The amount of

normetanephrine was found to be greater in the atrophied glands after protriptyline pretreatment than with no treatment. The data show the relative importance of the protriptyline-sensitive uptake mechanism and the intraneuronal deamination versus extraneuronal metabolism of noradrenaline.

Protriptyline reduced the ability of the slices to retain dopamine and noradrenaline in normal glands. The reduction was significantly higher for noradrenaline than for dopamine. In the atrophied glands protriptyline reduced the retention of dopamine to the same extent, whereas that of noradrenaline was less affected. The data from this study suggest that noradrenaline has a greater affinity than dopamine for the protriptyline-sensitive uptake mechanism of the adrenergic neurons.

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Activities of Monoamine Metabolizing Enzymes in Rat Salivary Glands A Comparison between Tissue Slices and Homogenates

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The metabolism of catecholamines has been studied for many years by using tissue slices or homogenates as model systems. By means of these techniques it has been possible to gain information about the properties of the different enzymes involved in the biosynthesis and metabolism of catecholamines. Monoamine oxidase (MAO) is one of the enzymes, which have been extensively studied in this way (for review see e.g. PLETCHER, GEY & ZELLER 1960). This enzyme can be effectively inhibited by e.g. nialamide. However there are certain differences between slice and homogenate techniques. Enzymes involved in the biosynthesis and metabolism of monoamines have different cellular and subcellular localizations. The different compartments of the enzymes are separated from each other by biological membranes. The substrates may have different abilities of penetration through these membranes. Furthermore there may be active transport mechanisms, which concentrate substrates within the cells. For instance, there is an amine concentrating mechanism at the level of the nerve cell membrane (HILLARP & MALMFORS 1964, HAMBERGER & MASUOKA 1965) and the slices, but not the homogenates, maintain the ability of taking up and concentrating the amines. This mechanism can be inhibited both *in vivo* and in the slices by a group of drugs, amongst which protriptyline is one of the most potent inhibitors (CARLSSON & WALDECK 1965, CARLSSON *et al* 1966, JONASON & RUTLEDGE 1968). In contrast to homogenates, the activity of intraneuronal localized MAO can be indirectly inhibited in slices by protriptyline (JONASON & RUTLEDGE 1968). In the slice preparations the different cellular and subcellular localizations of enzymes are normally preserved. In the homogenates, the cell mem

branes are abolished and the different enzymes are all localized in the suspension without any limiting membranes. The present study was performed in order to gain further information about the differences in enzyme activities - primarily in MAO activity - between rat salivary gland slices and homogenates.

Material and Methods

Male Sprague-Dawley rats weighing about 180 g were used in this study. Some of the animals were treated with nialamide 500 mg/kg i.p. 2 hours before death. The animals were killed by bleeding out under light chloroform anaesthesia. The submaxillary and sublingual glands were removed on both sides and weighed. The left glands were immediately sliced. In general, about 10 slices were obtained from each gland. The total amount of slices obtained from one gland was inserted into a single incubation flask containing 5 ml Krebs-Henseleit solution. The right glands were homogenized in 1 ml Krebs-Henseleit solution by a glass homogenizer and the homogenates were transferred into incubation flasks. Another 4 ml of the incubation medium was added to these flasks. The slices and the homogenates were preincubated for 10 min. at 37°C in a 95 per cent O₂ - 5 per cent CO₂ atmosphere. Then 3.670×10^{-12} moles of C¹⁴-dopamine (C¹⁴-DA) (46.5 ± 10^{-9} curies) or H³-noradrenaline (H³-NA) (6.66×10^{-7} curies) were added and the flasks were incubated for an additional 20 min. Control samples were prepared by the addition of 2 ml 2 N HCl to the flasks before the preincubation period. All incubations were terminated by the addition of 2 ml 2 N-HCl.

Procedures used in this study have been described in detail in previous publications (RUTLEDGE & JONASON 1967; JONASON & RUTLEDGE 1968) and thus will be presented here only in general terms. DA and NA were isolated by desorption onto a Dowex 50 column, from which these amines were eluted in separate fractions. When methoxytyramine (MTA) and normetanephrine (NM) were determined, the neutralized extracts were first passed over alumina, where the two catecholamines, DA and NA, were adsorbed. The alumina effluent, containing MTA and NM, was then passed over a Dowex 50 column and the two amines were eluted in separate fractions. The amine eluates were freeze-dried and the radioactivity was measured by the liquid scintillation technique. Recoveries of the amines were determined in each sample by adding carrier substances immediately on termination of the incubation and quantitating the amount remaining in the eluate after the separation procedures. The amount of amines recovered was quantitated by the fluorescence emitted at 335 nm on activation at 280 nm. When the total amount of the deaminated catabolites was determined the extracts were passed over Dowex 50 and the catabolites in the effluents were extracted with ether. The ether volumes were reduced to dryness, after which the scintillation counting fluid was added and then the radioactivity determined in a Packard Tri-Carb scintillation apparatus.

The values are expressed as means \pm standard errors of the mean (S.E.M.) and are presented as moles $\times 10^{-12}$ per flask obtained after a 20 min. incubation period. The values of the amines were calculated by correcting for efficiency, recovery, aliquot factors and the specific activity of the precursor. The values of the combined add and acetal catabolites were calculated in the same manner except that recovery values could not be assessed; thus no correction for this variable was made. All values represent amounts of catabolites in the media as well as that in the tissue. The P values were calculated by Student's t-test.

Results

Metabolism of DA and NA in rat salivary gland slices and homogenates

The results are presented in table 1. Normally very large amounts of DA were metabolized in both tissue homogenates and slices of the salivary glands during the 20 min incubation period. The levels of unmetabolized DA were considerably lower than those of NA after this time interval. As seen from table 1 the amounts of DA and NA remaining after 20 min. incubation were significantly lower in the homogenates than in the slices ($P < 0.001$). When salivary gland slices were incubated with DA small amounts of NA were detected. In salivary gland homogenates the values of NA were less than zero when the control values were subtracted. However it is probable that some NA is actually synthesized from DA also in the homogenates. The relatively large control values of C^{14} -NA (which have been subtracted from the experimental values) are probably due to partial contamination with the radioactive precursor (1-2 per cent of DA in the control) during the separation procedures. In contrast to the control, only small amounts of unmetabolized DA are normally found in the homogenates after 20 min. incubation. Two per cent of this small value yields about 10×10^{12} moles as the actual control value of C^{14} -NA in this case. Therefore, it is probable that some NA is also synthesized from DA in the homogenates.

Large amounts of acid and neutral catabolites were isolated when DA was used as substrate. The levels of these deaminated catabolites in salivary gland slices were about twice those found in the homogenates ($P < 0.001$). When NA was used as the substrate much smaller amounts of acid and neutral catabolites were detected. However the amounts of deaminated catabolites found after incubation of salivary gland slices with NA were larger than those found after incubation of homogenates with NA ($P < 0.025$). The levels of the 3-O-methylated product of DA, namely MTA, were found to be considerably higher in the slices than in the homogenates ($P < 0.001$). When NA was used as substrate large amounts of NM were detected after incubation with salivary gland slices. About one fourth of these amounts was found in the homogenates ($P < 0.001$). Normally no NM was detected after incubation with DA. The relatively large control values of H^3 -NM are due to partial contamination with the radioactive precursor H^3 -NA.

Effect of nialamide on the metabolism of DA and NA in rat salivary gland slices and homogenates

As seen from table 1 pretreatment of the rats with nialamide 500 mg/kg 2 hours before killing the animals increased the amount of unmetabolized

Table 1

			Normal		Nialamide 500 mg/kg 2 hours		Control	
			Slices	Homogenates	Slices	Homogenates	Slices	Homogenates
5670 $\times 10^{-12}$ moles C^{14} -DA	DA	Mean	1391	485	3346	1042	5192	5728
		S.E.M.	93.8	99.0	190	94.5	501	258
		n	13	11	5	10	5	2
	NA	Mean	143 (55.0)	49.0 (0)	261 (173)	145 (36)	88.0	109
		S.E.M.	8.9	3.9	20.0	21.6	15.8	28.8
		n	13	13	4	8	12	10
	MTA	Mean	201 (195)	42.1 (33.3)	652 (646)	137 (128)	6.4	8.8
		S.E.M.	8.2	3.1	44.1	14.2	1.1	0.9
		n	5	6	4	3	3	3
	NM	Mean	17.7 (0)	7.2 (0)	105 (84)	24.7 (2.3)	21.0	22.4
		S.E.M.	2.9	1.0	18.8	4.0	7.7	1.2
		n	5	6	10	2	4	3
10 $^{-12}$ moles H^3 -NA	NA	Mean	1476 (1470)	713 (708)	18.2 (11.9)	8.9 (4.1)	6.3	4.8
		S.E.M.	65.9	54.3	3.1	3.1	3.5	3.0
		n	14	14	5	4	5	5
	NM	Mean	2682	1755	3780	3494	4685	4915
		S.E.M.	107	88.8	18	239	170	61
		n	13	13	4	4	6	7
	NM	Mean	409 (373)	130 (93.3)	791 (755)	190 (155)	36.3	34.7
		S.E.M.	17.3	13.1	44.0	15.3	5.2	6.8
		n	6	6		3	3	3
	Acid and neutral catabolites	Mean	15 (204)	161 (149)	18.2 (7.1)	15.4 (1.1)	11.1	14.3
		S.E.M.	17.4	10.4	0.2	0.4	0.8	6
		n	13	13	4	4	5	5

Homogenates or slices of rat salivary glands were incubated for 20 min. with 5670 10^{-12} moles of either ^{14}C -dopamine (46.5×10^{-9} curies) or 3H -noradrenaline (6.66×10^{-7} curies). Some animals were injected with nialamide 500 mg/kg 2 hours before the animals were killed. Control correspond to samples in which 2 ml of 2 M HCl were added to the incubation fluid before the incubation. The recoveries of the compounds were (based on at least 11 values) dopamine (DA) $76.6 \pm 1.6\%$, noradrenaline (NA) $88.8 \pm 1.2\%$, methoxy tyramine (MTA) $86.6 \pm 4.1\%$, and normetanephrine (NM) $82.1 \pm 1.4\%$. The values of DA, NA, MTA and NM but not the values of the acid and neutral catabolites were corrected for recovery. n represents the number of experiments. S.E.M. represents the standard error of the mean. Values are presented as moles 10^{-12} and represent amine or catabolite in the media as well as that in the tissue. Values in brackets represent values in which the control values have been subtracted.

DA (using DA as substrate) and NA (using NA as substrate) in the slices ($P < 0.001$). In the homogenates NA was also increased after this pretreatment ($P < 0.001$) whereas there was no significant increase of DA. Acid and neutral catabolites were reduced to negligible amounts when both DA and NA were used as substrate. When salivary gland slices and homogenates were incubated with DA the level of MTA was increased more than three fold ($P < 0.001$). However the amounts of MTA were found to be considerably higher in the slice preparations than in the homogenates ($P < 0.001$). After pretreatment of the animals with nialamide small amounts of NM from DA were detected in the slices ($P < 0.01$ as compared to the normal values). When NA was used as substrate pretreatment of the animals with nialamide markedly increased the amounts of NM both in the homogenates and in the slices, the amount of this 3-O-methylated amine being markedly larger in the latter than in the former preparation. Noradrenaline was synthesized from DA after nialamide treatment in both homogenates and slices. The amount of NA found in the slices after MAO inhibition was increased more than three times as compared to normal ($P < 0.001$) but was still markedly higher than in the MAO-inhibited homogenates.

Discussion

It is obvious from this investigation that the deamination reactions proceeded at a higher rate in the slices than in the homogenates. This is true regardless of whether DA or NA was used as the substrate. After pretreatment with the MAO inhibitor nialamide, only negligible quantities of deaminated catabolites were detected. By homogenization, an important property of the adrenergic nerve cells disappears, namely the ability of adrenergic nerve cells to concentrate amines within the cells (HILLARP & MALMFORS 1964). This mechanism concentrates the substrate for intraneuronal MAO within the neuron. Thus, loss of this mechanism might result in a slower deamination rate. Using slices appears to be a more physiologically reliable technique than using homogenates. When NA was used as the substrate relatively small amounts of acid and neutral catabolites were detected. This is partly a reflection of the fact that the major deaminated catabolites of NA are the phenolic glycols (RUTLEDGE & JONASON 1967 JONASON unpublished) and are not extracted into ether as well as the major catabolites of DA which are phenolic acids. However it is known from previous investigations that the deamination of NA is considerably slower than that of DA (JONASON & RUTLEDGE 1968 JONASON unpublished).

The activity of catechol-O-methyl transferase (COMT) also appeared to be higher in the slices than in the homogenates. The levels of MTA and NM (using DA and NA as substrate) were considerably higher in the slices than in the homogenates (6 and 4 times higher respectively). Almost the same difference was observed after nialamide. However after this pretreatment the levels of these 3-O-methylated amines were increased two or three times. MAO inhibition results in higher substrate concentrations for COMT which might partly explain the high levels of these 3-O-methylated products. Furthermore, treatment with nialamide inhibits the further degradation of MTA and NM by MAO which also results in higher amounts of 3-O-methylated amines. It is known from previous investigations (JONASON unpublished) that the major catabolite of exogenously administered NA in rat salivary glands is NM whereas those of DA are the phenolic acids 3,4-dihydroxyphenylacetic acid and homovanillic acid. As seen from table 1 the level of NM from exogenously added NA is markedly higher than that of MTA from DA. The reason for the relatively low activity of COMT in the homogenates might be lack of cofactors in the non-supplemented homogenates, or the presence of an inhibitory substance.

NA was synthesized from DA by salivary gland slices and probably also small amounts by the homogenates. After pretreatment with nialamide the amount of NA was increased about three times and small amounts of this amine were also found in the homogenates. The reason for this might be the increased substrate concentration for the NA synthesizing system due to treatment with the MAO inhibitor or inhibition of the further degradation by MAO. This higher level of NA results in an increase of NM the further metabolism of which is inhibited. It can be argued that large amounts of metabolites of newly-synthesized NA from DA might be present in the deaminated fraction. This might be true normally but not after nialamide treatment. In previous investigations (JONASON unpublished) it has been shown that deaminated metabolites of newly synthesized NA (primarily glycols) constitute less than two thirds of the total NA synthesis of the slices.

The amounts of unmetabolized DA and NA in the homogenates are low. The reason for this is probably the presence of other catabolites which were not analyzed e.g. oxidation products or conjugates. AXELROD (1964) has shown that an enzymic oxidation of adrenaline and related catecholamines to adrenochrome occurs in the homogenates of salivary glands. If oxidation products are formed from DA and NA in the salivary glands *in vitro* it is obvious that this oxidation reaction occurs more easily in the homogenates than in the slices. The reason for the difference between homogenates and slices might be that in the slice preparation the amines

have to pass through intact cell membranes before reaching the enzyme provided that the enzyme is localized intracellularly

The present study shows the importance of intact cell membranes in the metabolism of catecholamines. The rate of deamination, 3-O-methylation and NA-synthesis is higher in slice-preparations - with intact cell membranes - than in homogenates. On the other hand the data also indicate that there is an unknown metabolic pathway of catecholamines in the homogenates of salivary glands.

It is known that only about one third of the MAO activity is localized intraneuronally in the adrenergic nerves of rat salivary glands (ALMGREN *et al* 1966). However atrophy (which reduces the MAO activity by about two thirds) does not significantly reduce the total amount of deaminated catabolites in contrast to treatment with protriptyline (JONASON 1968). These data indicate that the intraneuronal part of the enzyme (one third of total activity) is of great significance. From table 1 it is obvious that the rate of deamination is higher in slices than in homogenates in spite of the fact that there are no limiting cell membranes in the homogenates. The data supports the view that there is a real "pump" mechanism in the cell membrane which concentrates the substrates within the adrenergic nerves resulting in higher amine concentrations, available for intra neuronal MAO

Summary

Slices and homogenates of rat salivary glands were incubated with C^{14} -dopamine or 3H -noradrenaline. Large amounts of deaminated catabolites were isolated when dopamine was used as substrate. When slices and homogenates were incubated with noradrenaline, smaller amounts of acid and neutral catabolites were found but by using both dopamine and noradrenaline as substrates, larger levels of deaminated metabolites were detected in the slices than in the homogenates. The amounts of ^{14}C -methoxytyramine and 3H normetanephrine were also found to be higher in the slices than in the homogenates. Noradrenaline synthesis from dopamine occurred in the slices and probably also in the homogenates. Considerably lower quantities of unmetabolized dopamine and noradrenaline were detected in the homogenates as compared to the slices, indicating the presence of other catabolites in the homogenates, e.g. oxidation products or conjugates. Nialamide pretreatment reduced the deaminated ^{14}C and 3H -catabolites to negligible amounts, whereas the levels of ^{14}C -methoxytyramine, 3H normetanephrine, ^{14}C -noradrenaline and unmetabolized substrates were increased. Larger quantities of all of these amines were also found in the slices than in the homogenates after

Methods

Implantation of permanent ventricular cannulas.

Drugs were administered in the lateral cerebral ventricles through chronically implanted cannulas in ten adult beagles of both sexes (weight 12–16 kg). The implantation was carried out under halothane anaesthesia and employing aseptic precautions. The cannulas (outer diameter 0.6 mm) were inserted stereotactically through a burr hole in the parietal bone 10 mm in front of the interaural line, 9 mm laterally to the midsagittal plane and perpendicular to the plane through the interaural line and infra-orbital edge. The blunt cannulas were gently pushed into the brain until a rich flow of CSF was obtained. When in position, the cannulas were fixed to the cranial vault with dental cement which was anchored to the parietal bone with two screws 1.5–2.0 cm apart. The top of the cannula was closed with an injection cap. For the registration of EEG two thin cables were drawn from the bone screws subcutaneously to the interscapular space. The scalp was sutured around the top of the cannula which was left free.

The dogs used in the experiments showed no unusual features following the operation. The wound was kept dry and free from infection (Fig. 1). Experiments were not performed until one week after the implantation, and the dogs were allowed to become accustomed to the experimental situation for 6–12 hrs before the trials were commenced. Between two and six experiments were performed on each dog. The intraventricular injections had volume of 0.25 ml, and pH was adjusted to 7.4 with solid sodium bicarbonate.

Histological examinations of the brain were done in three dogs. In all dogs a few siderophages were found along the cannula track and some slight perivascular histiocytic proliferations at the site of entrance of the cannula into the lateral ventricle. However some proliferations were also seen close to the ependyma in the contralateral and third ventricle. Limited subependymal softening of the brain substance with numerous glitercells were seen in one dog, which had received 0.25 ml hyphae (®) (sodium amidoarizate NFN) intraventricularly one day before autopsy. Meningeal reactions were never detected.

Registration and evaluation of symptoms.

Gross behaviour was observed through one way screen in a special observation cage (2 x 1.5 m). The cage was air conditioned and the temperature was kept at between 18–20 °C. In half of the experiments the behaviour of the dogs was recorded on video tape for more detailed evaluation.

Special attention was paid to the symptoms comprising the anticholinergic syndrome: ataxia, unresponsiveness and non-avoidance i.e. walking into objects without retreating. The symptoms were rated as follows. Stiffness of gait was called slight ataxia, stumbling = moderate ataxia and falling including inability to rise = severe ataxia. Moderate unresponsiveness was recorded when the dogs did not react when called and severe unresponsiveness when they did not react to physical contact. Non-avoidance was called moderate when the dogs walked into objects and severe when trying to force them or not aware of them.

In addition rectal temperature and heart rate were recorded. The latter was continuously telemetered and recorded on polygraph while the rectal temperature was measured at regular intervals. The two channel telemetering unit (Telmedix, Southampton, Pennsylvania,

) Our thanks are due to Tony Krusajević, VMD State Veterinary Medical Institute, Stockholm.



Fig. 1 Dog wearing harness with telemetering unit for transmitting EEG and ECG. Venous catheter in the hindleg. The top of the chronically implanted cannula is shown.

USA, $5 \times 7.5 \times 11$ cm, weight 0.6 kg) was carried in a special harness placed on the back of the dogs (Fig. 1).

The effects of the drug on the EEG were also investigated in eight experiments on four dogs. The EEG was telemetered on the second channel as the potential difference between the screws in the cranial vault, and recorded on a polygraph. Normal EEG had an amplitude of 5–75 μ v under these conditions. The reliability of the EEG unit was checked as to frequency amplitude and distortion with a sinus wave generator both before and after each experiment.

Systemic absorption following the two routes of administration.

Plasma concentrations of atropine and methylatropine were determined following the administration of the tritium-labelled drugs. Blood samples were withdrawn through catheters introduced into limb vein before starting the experiment. When the distribution

) Generally tritiated atropine was purchased from the Radiochemical Centre, Amersham. Radioactive methylatropine was synthesized by quaternization with unlabelled methylbiodide (ALABANT *et al.* 1968 b).

of the drugs | the CSF was studied, the dogs were anaesthetized with halothane and the CSF collected from the different compartments as described earlier (ALBANUS *et al.* 1968). Extraction and assay of radioactivity | plasma, CSF and urine were performed as previously reported (ALBANUS *et al.* 1968 & 1969). Following subcutaneous injection of atropine and methylatropine, less than 10% and 20% respectively are metabolized in the dog within 2 hrs (ALBANUS *et al.* 1968 & 1969). Drug concentrations were therefore calculated from the specific activity of the material and the concentration of radioactivity in the plasma and CSF.

Results

1 Effects of subcutaneous injection of atropine

Heart rate – All the doses studied (0.1 to 0.5 mg/kg of atropine sulphate) produced a marked rise in heart rate and the disappearance of the respiratory cardiac arrhythmia. The tachycardia started 5 min. after the injection and reached a maximum after about 15 min. Thereafter the heart rate slowly returned to normal during the following 2–5 hrs, depending on the dose administered. The degree of tachycardia and its duration were both dose dependent. The effect of 0.5 mg/kg is seen in fig. 2 and table 1. The anticholinergic syndrome appeared in all six experiments with 0.5 mg/kg, but not with the lower dose of 0.3 mg. Ataxia and unresponsiveness occurred almost simultaneously after a mean time of 43 (30–50) min. Non-avoidance appeared somewhat later after approximately 60 (45–70) min (table 1). The ataxia developed in a highly characteristic sequence firstly stiffness in the hindlegs on walking, followed a few minutes later by

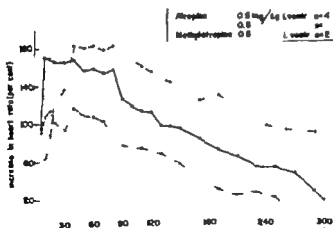


Fig. 2. The effects on heart rate following intracerebroventricular and subcutaneous injection of atropine and methylatropine. Per cent of the mean heart rate during at least one hr prior to the injection. n = number of dogs.

Table 1

Comparison of symptom criteria used with atropine and methylatropine *folio* log Intracerebroventricular and subcutaneous Injection (0.5 mg/kg as tropine sulphate and methylatropine nitrate).

	1 - Ataxia			Convulsive seizures		Rectal temperature increase		EEG change		Heart frequency increase		Vomiting Defecation Micturition	
	2 - N	3 - Unresponsiveness		x/y	T	x/y	T _{max}	x/y	T	x/y	T _{max}	x/y	T
Atropine subcutaneously	1	6/6	45	0/6		0/6	-	3/3	35	6/6	15	0/6	-
	2	6/6	61										
	3	6/6	40										
Atropine intraventricularly	1	6/6	40	3/6	88	6/6	60	3/3	40	6/6	10	6/6	4
	2	4/6	55										
	3	6/6	40										
Methylatropine intraventricularly	1	2/2	250	2/2	-	2/2	90	2/2	250	3/3	40	3/3	3
	2	0/2											
	3	2/2	250										

x/y Number of animals responding/ number of animals tested

T Mean time (min.) until response.

T_{max} Mean time (min.) until maximum effect.

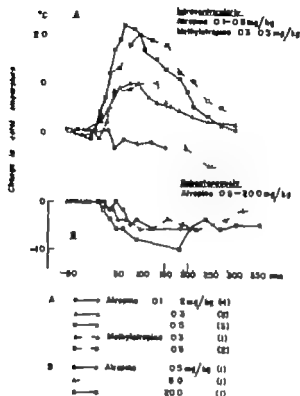


Fig. 3 Changes in rectal temperature following intracerebroventricular and subcutaneous injection of atropine and methyldatropine.

stumbling and slipping. Later the dogs rocked on their forelegs and sometimes they knelt on their forelegs with elevated hind quarters still swaying. The stumbling became progressively worse and the animals fell to the floor sometimes apparently unable to rise.

The symptoms were most pronounced after 65-85 min., gradually fading during the following 4 hrs (table 1). Although these symptoms are reproducible and characteristic, they may vary in intensity from one minute to the next. At the height of the intoxication, the dogs also showed periods of rather well coordinated hyperactivity during which they ran around in their cages and barked continuously.

EEG - In one experiment with an atropine dose of 0.3 mg/kg an increase in amplitude of about 2-fold, and a slight decrease in frequency occurred after 60-70 min. and lasted for one to two hrs. Following 0.5 mg/kg, a 5 to 10-fold increase in amplitude was registered after 30-40 min. About 10 min. later the normal frequencies of 40 and 20 cps gradually

fell to 20 and 10-12 cps. In addition, a new frequency of 1-4 cps was sometimes recorded. The most obvious changes lasted for 70-180 min. but did not disappear completely until 7 to 9 hrs after injection. The effects on behaviour and EEG showed an approximately simultaneous development.

Rectal temperature - Subcutaneous injection of 0.5, 5 and 20 mg/kg produced a decrease in rectal temperature (0.5-1.0°). The results are summarized in fig. 3B. It will be seen that the maximum effect is obtained after about 1.5 hr and that, even after 4 hrs, the temperature has still not returned to normal.

2. Effects of intraventricular injections of atropine

Following intraventricular injection, a number of symptoms were noted in addition to those described in the preceding paragraph. The doses of atropine tested in this series of experiments ranged from 0.1 to 0.5 mg/kg. Control experiments with intraventricular saline were also performed and no effects on the recorded symptoms could be detected.

Heart rate - The time course of the tachycardia was the same as that following subcutaneous injection but the increase was much higher (cf fig. 2).

The anticholinergic syndrome was also elicited following intraventricular administration. Even using this route of administration, 0.5 mg/kg of atropine was needed to produce the typical syndrome. In one experiment out of seven, this was induced by 0.3 mg/kg. The time until onset and the course of the syndrome were identical with that following subcutaneous injection (see table 1).

The EEG changes showed the same pattern and time course as in the case of a subcutaneous injection of the same dose.

Temperature - 0.1-0.2 mg/kg caused a small decrease in rectal temperature, while 0.3 and 0.5 mg/kg caused a rapid, dose dependent increase (fig. 3A). After 0.3 mg/kg, an average maximum increase of 1° was obtained within 60-90 min. 0.5 mg/kg gave a maximum increase of 2.5° in about 60 min. The effect gradually decreased during the next 4-5 hrs.

Convulsive seizures occurred in six experiments out of seven after 0.5 mg/kg. In one dog, convulsions also occurred after 0.1 and 0.3 mg/kg. The first seizure was seen 88 (30-150) min. after the injection. In five out of six experiments, the attacks were preceded by head shaking and chewing. Head shaking was also seen in two dogs after 0.3 mg/kg without any subsequent fit. The seizures were strikingly uniform and began with head shaking and turning the head to the side opposite to that of the injection, after which the dogs fell down on their side with stiff extended legs.

Within 10 sec., this phase turned into a stage of apparently well-coordinated running movements with all four legs. After the fit the animals remained recumbent for 1-15 min.

Panting was a constant symptom during the height of the intoxication.

Micturition occurred in most of the dogs with the lower doses, but in only one of 6 experiments with 0.5 mg/kg, the first occurred 7 (4-12) min. after injection.

Vomiting was seen after all doses although, after 0.1 and 0.2 mg/kg, only in about half of the dogs. Vomiting started after a mean delay of 5 (2-9) min. after injection and lasted for about 15 min. It was associated with other symptoms of nausea such as retching and restlessness.

Defaecation was seen in about half of the dogs following all doses and occurred 15 (8-40) min. after the injection.

3 *Effects of intraventricular injection of methylatropine*

Methylatropine nitrate (0.3 and 0.5 mg/kg) was injected into the right ventricle in three dogs. Symptoms similar to those following the administration of atropine by the same route occurred, but those characteristic of the anticholinergic syndrome appeared very late and were not impressive (table 1).

Heart rate increased more slowly than after intraventricular administration of atropine but the maximum increase was of the same order of magnitude (fig. 2).

Gait was normal until 4-5 hrs, when there was again a *slight ataxia* with stumbling, fits of hyperactivity, unusual shyness and a somewhat impaired avoidance reaction. These symptoms lasted for as long as two days but were never as pronounced as the anticholinergic syndrome seen after atropine. A symptom peculiar to methylatropine (0.5 mg/kg) was walking in circles which directed contralaterally to the side of injection. The circling started after 25-65 min. was most pronounced between 70-90 min. and then subsided during the next hour. No nystagmus was observed even during the most vigorous circling.

No changes in *EEG* were seen until 4-5 hrs after the injection when the dogs showed an abnormal gross behaviour. The changes then resembled those seen after atropine but were less pronounced.

The increase in *temperature* was the same as after atropine administered intraventricularly (fig. 3A).

Convulsive seizures were seen after 7-265 min. The seizures closely resembled those following atropine but the onset differed in that the dogs twisted their whole body to the side opposite to the injection. After the

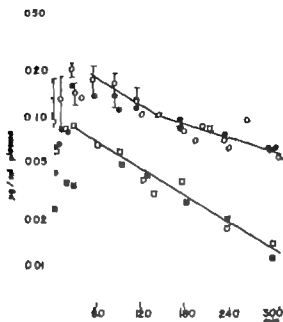


Fig. 4 Absorption of atropine into plasma of dogs following subcutaneous and intraventricular administration of the labelled drug (expressed as atropine sulphate)

- subcutaneously } 0.3 mg/kg
 ● intraventricularly }
 □ subcutaneously } 0.3 mg/kg
 ■ intraventricularly }

Vertical bars indicate max. and min. values (five dogs).

seizures, the dogs appeared unresponsive and showed a slightly ataxic gait for less than one min.

In all cases vomiting and defaecation were seen after 4–6 and 3–5 min. respectively (table 1).

4 Relationship between plasma concentrations and the anticholinergic syndrome

The results presented in the previous paragraphs show that both threshold dose and time course of the anticholinergic syndrome were practically the same following subcutaneous and intraventricular administration of atropine. When the plasma concentrations of ^3H -atropine were measured it was found that the plasma concentration curves were very similar following both routes of administration (fig. 4). The figure also indicates that plasma concentrations of about $0.1 \mu\text{g/ml}$ are needed to produce the syndrome.

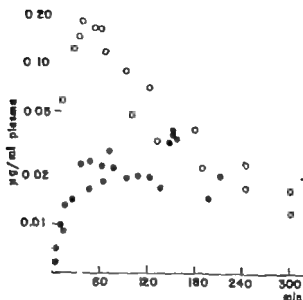


Fig. 5 Absorption of methylatropine into plasma following subcutaneous (O) and intraventricular (●) administration (0.5 mg/kg expressed as methylatropine iodide).

A similar comparison of plasma concentrations following the two routes of administration of methylatropine is shown in fig. 5. In this case a very large difference (up to 10-fold) was found.

5 CSF distribution of injected material.

In order to estimate the distribution of the injected material within the CSF 0.25 ml contrast medium (hypaque ® 90/6) was injected into the right lateral ventricle of two anaesthetized dogs. As seen in fig. 6 the radio-opaque material was distributed throughout the posterior part of the injected ventricle and the third ventricle within 12 sec. After 1 min., contrast was also seen in the rostral horn of both lateral ventricles and was visible in the fourth ventricle. After 10 min. the contrast in the aqueduct and fourth ventricle faded and also began to diminish in the injected ventricle. No signs of disappearance were seen in the third ventricle at that time.

The uneven distribution was verified by injecting ^3H -atropine into one of the lateral ventricles and collecting 0.1 ml samples of CSF from both

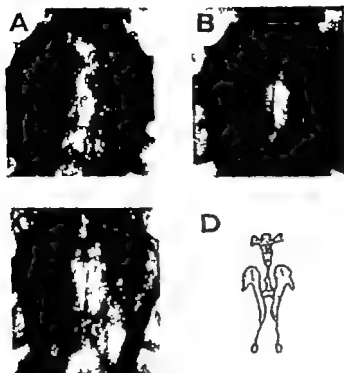


Fig. 6. X-ray photographs of the ventricular system at different times following intra-ventricular injection of hypaque @ 90% (0.25 ml in the right lateral ventricle). A 12 sec. B 1 min. C 10 min. after injection. D Drawing of a vinylite cast of the ventricular cavities (after FITZGERALD 1961).

lateral ventricles, the cisterna magna and the epicerebral subarachnoid space at different intervals. The disappearance of ^3H -atropine was also compared with that of inulin, since the latter drug is known to be cleared from the ventricular system almost exclusively by the bulk flow of CSF RALL *et al* (1962). A ratio of 6 was found between the concentrations of atropine in the two lateral ventricles after 2 hrs and the ratio remained practically constant during the following 8 hrs. The corresponding figure for inulin was 3. The difference in the rate of disappearance of the two drugs from the injected ventricle is seen in fig. 7. From 1 to 6 hrs, the half life of atropine is 100 min. and thereafter 240 min. while the half-life for inulin is 240 min. from 2 to 10 hrs. It is also seen that a considerable amount of atropine has been cleared from the CSF during the first 3 hrs of its passage to the cisterna magna giving a 50-fold difference in the concentrations of the drugs.

In our experiments, the anticholinergic syndrome was elicited both after subcutaneous and intraventricular injection and no difference in the minimum effective dose or time course could be demonstrated. In addition, the syndrome was produced at approximately the same plasma concentrations of atropine following both routes of administration. These results strongly suggest that the syndrome is elicited when the drug reaches the brain via the blood. However there are a few observations which indicate that, following intraventricular administration when the CSF contains very high concentrations of atropine (5000 times the plasma concentration), a direct penetration from the CSF into the brain could elicit the syndrome. In Edery's experiments in which atropine was injected into the third ventricle, the minimum effective dose was at least 5 times lower than that in our own experiments where we injected atropine into one lateral ventricle, and where the time course was more rapid. Already 5 min. after the injection the above author noted the first symptoms typical of the anticholinergic syndrome. This discrepancy may indicate that the symptoms are elicited from structures lining the third ventricle. The X-ray studies on the movement of contrast medium injected into the ventricular system by MCCARTHY & BORISON (1967) and by us, also show that the injected contrast is retained in the third ventricle for a considerable time. Our experiments with intraventricularly injected methylatropine also indicate a direct effect since a weak syndrome is elicited after 4-5 hrs following intraventricular injection of 0.5 mg/kg but no effect is produced following the subcutaneous injection of 2.5 mg/kg (ALBANUS, unpublished results). It would be surprising if methylatropine but not atropine, was able to penetrate deep enough from the CSF into the brain to produce the anticholinergic syndrome. However the fact that atropine is very rapidly absorbed into the blood following injection into a lateral ventricle makes it improbable that a direct penetration is of major significance in the production of the anticholinergic syndrome.

It is interesting to note that intracerebroventricular injection of atropine is more effective in producing tachycardia than subcutaneous injection. Judging from the plasma concentration curves, the opposite relationship would be expected if a solely peripheral site of action is assumed. In a recent paper DONALD *et al* (1967) found evidence of a central component in the atropine induced tachycardia, which may explain our findings.

Circling behaviour has been reported following injections of various drugs into a lateral ventricle. Thus, tubocurarine produces circling to the ipsilateral side while DFP, neostigmine and GABA induce circling to the contralateral side (FELDBERG & SCHERWOOD 1954a & b; TRACZYKE 1959). It is somewhat confusing that methylatropine falls into the same category as DFP and neostigmine, i.e. circling towards the contralateral side, and

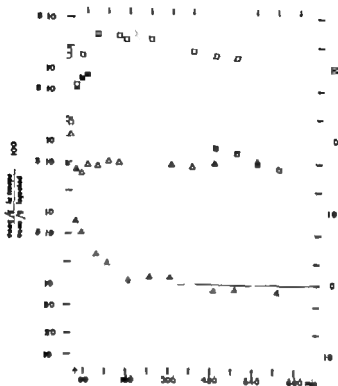


Fig. 7 Distribution of tropine and lanli in different compartments of the CSF flowing in lateral ventricle

- | | |
|-------------|--|
| (○) inulin | } lateral ventricle on the injected side |
| (■) tropine | |
| (△) lanli | } cisterna magna |
| (▲) tropine | |
| (+) tropine | in the epineural subarachnoid space. |

Discussion

The symptoms described in the preceding paragraphs fall into two major groups. The first group of symptoms was obtained with atropine following both routes of administration and consisted of the central anticholinergic syndrome, EEG-changes, and tachycardia. The other group of symptoms was obtained only following intraventricular injection of atropine and methylatropine. The symptoms in this latter group were hyperthermia vomiting, micturition, defaecation, and convulsive seizures.

As mentioned in the introduction it has been suggested that the symptoms constituting the anticholinergic syndrome may be elicited from structures lining the cerebral ventricles which could be reached by atropine after penetration from the CSF (EDERY 1962 FELDBERG 1963).

aid of the tritium labelled drug. A rapid absorption was demonstrated following both routes of administration and the plasma levels were approximately the same. Plasma concentrations of about 0.1 µg/ml produced a typical sequence of behavioural effects.

Measurements of the rate of disappearance of atropine in relation to mulin from different compartments of the ventricular system indicated that absorption occurs in the cerebral ventricles.

Experiments with methylatropine, which was found to be less efficiently cleared from the CSF to the blood and which does not produce the behavioural effects following subcutaneous injection, indicate that behavioural effects can be produced by penetration from the CSF into the brain. The symptoms appear after a delay of 4-5 hrs.

It is concluded that the behavioural syndrome is elicited from structures in the vicinity of the ventricles but that direct penetration from CSF to these brain structures is not important following subcutaneous injection. When very high gradients between CSF and brain occur as after intra ventricular injection, direct penetration may produce the central effects, but in the case of atropine the rapid absorption into the blood, outruns this process.

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On the Metabolic Disposition of Methylatropine in Animals and Man

By

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The efficiency of enteral absorption of quaternary anticholinergic drugs is a matter of debate, (HERXHEIMER & HAEFFEL 1966 MÖLLER & ROSÉN 1967 WICK 1967). Information has generally been gained by comparing oral and parenteral doses producing an identical effect. The results have differed with ratios from $1/5$ - $1/3000$ depending on the species and the effect studied (MÖLLER & ROSÉN 1967).

In the present paper results are presented on the absorption, metabolism, distribution and excretion of methylatropine obtained by means of the tritium labelled drug.

Methods

Tritium labelled methylatropine iodide was synthesized from generally tritium labelled atropine by quaternization in ethanol with unlabelled methyl iodide. 10 μ l methyl iodide was added to a solution of about 20 mg of tritium labelled atropine (Radiochemical Centre Amersham, spec. act. 245 mCi/mmol) in 40 μ l 96% ethanol. The reaction mixture was left at room temperature for 2 weeks. After dissolution in 30 μ l 96% ethanol of the viscous product formed, methylatropine was precipitated by the addition of 400 μ l dry ether. The yellowish precipitate formed after refrigeration for one night was isolated by centrifugation, dissolved in 40 μ l hot 96% ethanol and left overnight in the refrigerator. The crystalline mass formed was isolated, washed with ice cold ethanol and recrystallized four times from hot ethanol. After the last recrystallization the white crystals were washed with dry ether and dried overnight in vacuum over P_2O_5 at 40-45°. The identity and purity of the product was checked by high voltage electrophoresis in borate buffer of pH 10 together with commercial unlabelled methylatropine. The electropherograms were scanned in Packard strip scanner and the unlabelled compound visualized by spraying with modified Dragendorff reagent. The only radioactive impurity detected was small contamination with atropine (< 1%). The pharmacological effects of the labelled compound were compared with those of commercially available unlabelled methylatropine nitrate following subcutaneous admini-

stration to conscious dogs. No difference in the potency of the two preparations could be detected when the effects on heart rate and pupil size were measured (0.1 mg/kg).

Mouse experiments.

Adult CBA mice of both sexes were used. 6 mg/kg (3 mCi/kg) methylatropine iodide was injected intravenously or into the stomach. Blood samples were taken at intervals from the infraorbital plexus with a micropipette. Urine was collected on a piece of filter paper and the radioactivity in the paper strip was eluted with water. Groups of animals were killed by exsanguination after 30 min., 1 hr and 4 hrs and various organs rapidly excised, frozen in liquid nitrogen and ground to a fine powder. The tissue powder was extracted as described below.

Dog experiments.

The study was undertaken in ten beagles of both sexes weighing 10-15 kg. Methylatropine was administered subcutaneously in the back of the neck (50 μ Ci/kg corresponding to 0.1 ml/kg) and orally by instillation through a stomach tube. Different doses of methylatropine were obtained by adding unlabelled methylatropine nitrate. The heart rate was measured repeatedly throughout the experiment.

Blood and urine samples were taken at regular intervals.

At the end of the experiment the dogs were killed with pentobarbital sodium and the heart, mandibular gland, stomach and ciliary body rapidly removed for analyses. The stomach was thoroughly washed and a 5 cm segment adjacent to the pylorus was taken for analysis. From the heart, the right atrium and apex were analysed. After dissection the tissues were frozen in liquid nitrogen and ground to a fine powder in a mortar.

The content of the gall bladder was collected with a syringe, the volume measured and an aliquot taken for radioassay.

Assay of radioactivity in tissues and urine.

1 g of tissue powder (or 1 ml of heparinized plasma) was extracted with 2 ml acid ethanol (0.2 % acetic acid in 96 % ethanol) at room temperature for 30 min. and centrifuged. The pellet was then resuspended in 1 ml acid ethanol (0.15 % acetic acid in 70 % ethanol), extracted for 30 min. and centrifuged. The latter procedure was repeated once. The combined supernatants were concentrated to 1 ml and assayed by liquid scintillation in a Nuclear Chicago model 720 liquid scintillation counter. 0.5 ml of the extract were added 0.5 ml water and 14 ml of a scintillation solution containing 960 ml dioxane, 5.12 g PPO, 0.128 g POPOP and 102.4 g naphthalene. Quench corrections were made by the channels ratio procedure. The efficiency and reproducibility of the extraction procedure was checked by adding known amounts of radioactive methylatropine to heparinized plasma. The recovery was 95.5 % and the reproducibility ± 2.7 (standard error of the mean of 14 experiments).

Urine samples were assayed by adding 10-20 μ l of urine to 1 ml of water followed by 14 ml of the scintillation solution. Labelled metabolites in the urine were separated by paper chromatography and high voltage paper electrophoresis (see results).

Binding of methylatropine to plasma proteins was performed with the aid of centrifuging bags made of dialysis tubes (Union Carbide Co, Chicago, mean pore size 24 Å) containing 5 ml plasma in which had been added a known amount of radioactive methylatropine. The bottom of the centrifuge tube was covered with glass pearls and centrifugation (1200 g) continued for 2 hrs after which time about 0.5 ml of ultrafiltrate was produced. The ultrafiltrate was assayed for radioactivity as described above. Leakage of protein was checked according to the method of ZAMENHOF (1959) and found to be less than 5 %.

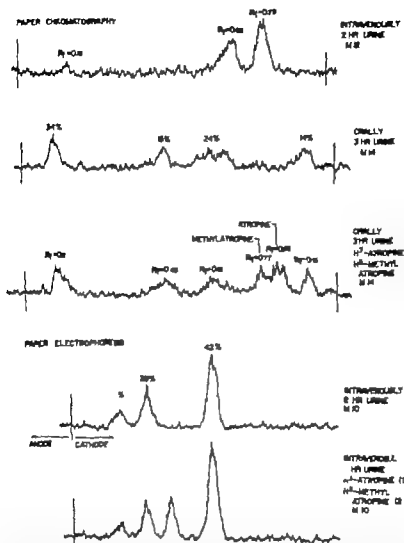


Fig. 1 Paper chromatography and high voltage paper electrophoresis of radioactive metabolites in the urine from mice following intravenous and oral administration of tritium labelled methylatropine. (The code M 11 denotes mouse number 12).

Results

Metabolic disposition of methylatropine in the mouse

Following intravenous injection of 6 mg/kg (3 mCi/kg) of tritium labelled methylatropine iodide, about 50% of the radioactivity is excreted in the urine within 2 hrs. Paper chromatography and high voltage electrophoresis of the urine reveal two major metabolites in addition to un-

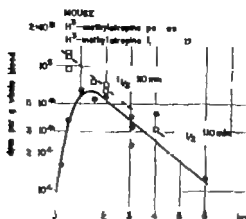


Fig. 2. Time course of the concentrations of radioactivity in the blood of mice following intra-venous and oral administration of tritium labelled methylatropine.

changed methylatropine (fig. 1). The R_f values in n-butanol, water:acetic acid (5:5:1) are 0.09–0.16 (metabolite I), 0.61–0.74 (metabolite II) and 0.77–0.84 (methylatropine). Following high voltage electrophoresis in borate buffer pH 10, all three compounds moved towards the cathode (metabolite I 0.31–0.34 and metabolite II 0.50–0.53 times the distance of methylatropine). In order to check the reliability of the separation technique, labelled or unlabelled methylatropine was always added to an aliquot of the urine and run simultaneously with the original sample (fig. 1).

Elution of the radioactive spots from one of the electropherograms showed that 42% of the radioactivity in a 2 hr urine specimen was in the

Table 1

Excretion of radioactivity in the urine and faeces in mice following oral administration of tritium labelled methylatropine. Figures represent per cent of administered dose.

Hr	M22		M23	
	Urine	Faeces	Urine	Faeces
4	29	27	29	34
48	< 1	2	< 1	1
72	1	< 1		< 1
T. total	30	30	30	35

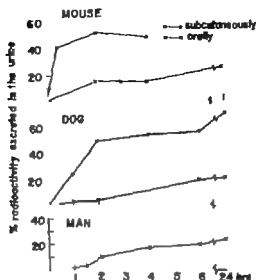


Fig. 3 Cumulative excretion of radioactivity in the urine following subcutaneous and oral administration of tritium labelled methylatropine in mice (7 animals), dogs (10) and man (1).

form of unchanged methylatropine 14% and 35% of metabolite I and II respectively

The half life of the radioactivity in the blood was 110 min. (fig. 2)

Following oral administration of the same dose, maximum blood concentrations of radioactivity are reached in 1 to 2 hrs (fig. 2). The half life of the radioactivity in the blood was the same as that following intravenous injection. The excretion of radioactivity in the urine and

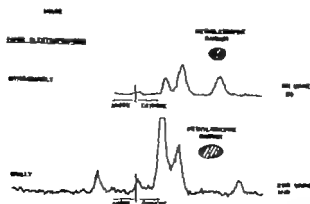


Fig. 4 High voltage paper electrophoresis of radioactive metabolites in the urine of mice following intravenous and oral administration of tritium labelled methylatropine. (The code M 20 denotes mouse number 20).

MOUSE

PAPER CHROMATOGRAPHY

ORIGINAL SAMPLE

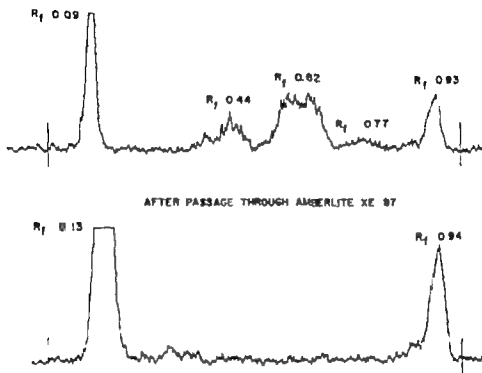
ORALLY
24 HR URINE
M19

Fig. 5 Separation of radioactive urinary metabolites on Amberlite XE 97 Paper chromatography of a urine specimen before and after passage of the column. (Mouse number 19).

faeces was followed during 72 hrs. As seen in table I not more than 60–65% of the radioactivity given could be recovered within this time. The major part was excreted within 24 hrs and equal amounts of radioactivity were found in the urine and faeces. After 3 hrs, 15% of the administered radioactivity was recovered in the urine (fig. 3). Elution of electropherograms showed that less than 2% was in the form of unchanged methylatropine. In addition to the metabolites present in the urine following intravenous injection, two other metabolites were found (fig. 1 & 4). These additional metabolites have the same R_f -values as the products formed by hydrolysis of methylatropine (R_f 0.40 and 0.91). The paper electrophoretic separation revealed that one of the new metabolites runs towards the anode (tropic acid) and the other towards the cathode (a distance 1.36 times that of methylatropine cf fig. 4).

Elution of the radioactive spots shows that metabolite I (R_f = 0.09–

Table 2

Concentration [radioactivity] different tissues in six mice following intravenous injection of tritium labelled methylatropine.

	Tissue concentration ratio					
	0.5 hr		2 hrs		4 hrs	
Blood	15	27	11	10	5	12
Brain	1	1	1	2	1	—
Heart muscle		118	23	21	3	5
Submaxillary gland	221	156	72	114	17	30
Pancreas	38	15	21	8	7	8
Adrenal	—	90	17	10	4	3
Lung	34	38	12	31	1	8
Stomach	23	14	7	6	5	7
contents	23	14	7	6	5	7
Ileum	—	44	37	33	75	44
contents	81	120	103	130	692	185
Liver	73	83	29	12	7	14
Bile	838	—	916	2197	—	792
Kidney	78	506	29	26	7	9

$$\frac{\text{dpm in tissue per g}}{\text{dpm injected per g}} \times 100$$

0.16) comprises 34% of the radioactivity in the urine metabolite II (Rf 0.60–0.65) 24%, metabolite III (Rf 0.41–0.46) 15%, and metabolite IV (Rf 0.91) 14% respectively (fig. 1). 13% of the radioactivity spotted on the chromatogram could not be found on elution.

The metabolites in the urine 2 hrs after intravenous and oral administration were also separated on an Amberlite XE 97 cation resin. A small column (45 × 5 mm) containing about 1 ml of the resin in a hydrogen form and equilibrated with 0.1 M NaH₂PO₄ was used. Following intravenous administration, about 20% of the radioactivity in the urine passed through the column while the corresponding figure following oral administration was 54%. After elution with 0.05 N HCl the remainder of the radioactivity left the column in one peak. By adding tritium labelled methylatropine to a urine specimen obtained after oral administration, it could be verified that all the methylatropine added was absorbed on the column and could be eluted with 0.05 N HCl. Fig. 5 shows a radioelectropherogram of a urine specimen before and after passage of the resin. As seen from the figure metabolites II and III and methylatropine are absorbed to the column whereas metabolites I and IV pass without absorption.

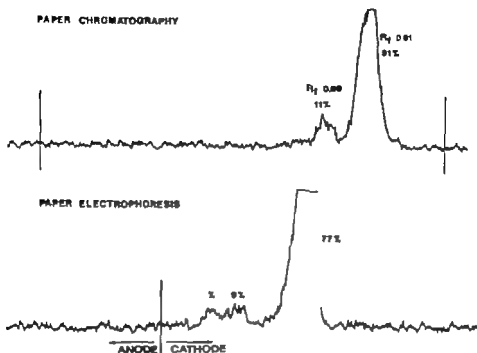


Fig. 6. Paper chromatography and high voltage paper electrophoresis of radioactive metabolites in the urine of two dogs 4 hrs after subcutaneous injection of tritium labeled methylatropine.

The distribution of radioactivity to various tissues was studied at different intervals following intravenous administration. The results are summarized in table 2. It is seen in the table that a rapid uptake of radioactivity occurs in the heart and submaxillary gland. After 30 min. the concentration in these organs is 5 to 10 times that in the blood. The high radioactivity in the submaxillary gland persists much longer than in the heart. The concentration in the brain is only 5-20% of the concentration in the blood.

Metabolic disposition of methylatropine in the dog

A marked difference in the plasma concentrations of radioactivity was found when the subcutaneous and oral administration were compared. Following 0.1 mg/kg (50 μ Ci/kg) subcutaneously a maximum plasma concentration is reached after 15 to 30 min. Oral administration of the same dose in a water solution gave a maximum plasma concentration after 2 hrs of $\frac{1}{4}$ th of the peak concentration reached following subcutaneous injection. The binding of methylatropine to plasma proteins was found to be less than 10%.

Table 3

Radioactivity in the gall bladder bile following subcutaneous and oral administration of ^3H -methylatropine in four dogs. The figures represent per cent of administered dose.

Route of administration	Killed after	
	1 hr	2 hrs
Subcutaneous	3.3%	6.5%
Oral	0.6%	1.5%

The excretion of radioactivity in the urine is seen in fig. 3. After 6 hrs, 60% of the radioactivity is excreted following subcutaneous injection while only 20% is excreted during the same period following oral administration. Within the next 18 hrs, an additional 10% is excreted in the case of subcutaneous injection but only 2% after oral administration. At 1 and 2 hrs there is a tenfold difference between the percentage excreted after subcutaneous and oral administration. Fig. 6 shows a radiochromatogram and an electropherogram of a urine specimen collected 4 hrs after subcutaneous administration. Elution of the radioactive spots showed that

Table 4

Concentration of radioactivity in different tissues in six dogs following subcutaneous and oral administration of tritium labelled methylatropine.

Tissue	Tissue concentration ratio					
	1 hr			2 hrs		
	0.1 mg/kg s.c.	1.0 mg/kg orally	0.1 mg/kg s.c.	0.2 mg/kg orally	1.0 mg/kg orally	2.5 mg/kg orally
Plasma	31.2	8.2	13.6	8.1	8.8	7.7
Maxillary gland	107	8.4	136	6.9	9.7	8.5
Heart, per	187	8.0	149	6.4	15.3	16.4
Heart, right atrium	212	10.9	122	6.7	16.9	16.4
Ciliary body	44.5	6.7	33.7	3.2	6.2	27.9
Stomach, antrum	115	118	129	84.8	162	132
Stomach, contents	7.9	7532	6.7	6746	4462	15878

$\frac{\text{dpm in tissue per g}}{\text{dpm administered per g}} \times 100$

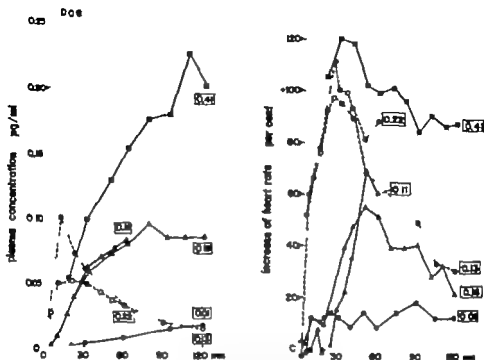


Fig. 7 Relationship between the concentrations of methylatropine (radioactivity) in the plasma and heart and the effects on heart rate. Heart rate was recorded in conscious dogs with the aid of a telemetering unit.

- — ○ subcutaneous injection of 0.1 mg/kg
- — ● oral administration of 0.1 mg/kg
- ▲ — ▲ oral administration of 1.0 mg/kg
- — ■ oral administration of 2.5 mg/kg
- denotes concentrations in heart tissue.

about 80% was in the form of unchanged methylatropine (fig. 6). No obvious difference in the electropherogram pattern was found in the urine collected after 6 hrs following oral and subcutaneous administration. The excretion of radioactivity in the bile is summarized in table 3. It will be noted that 4 times more radioactivity was found after subcutaneous than after oral administration.

Table 4 shows the concentration of radioactivity in different organs. When doses over 0.1 mg/kg were used, unlabelled methylatropine was added. One hr after the subcutaneous injection the mandibular gland, heart and stomach contains 3–7 times the concentration found in the plasma. After 2 hrs the concentration in these organs is 9–11 times the

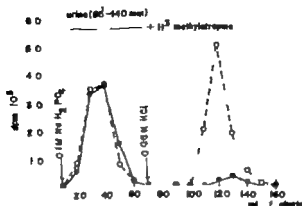


Fig. 8. Separation of radioactivity urinary metabolites on Amberlite XE-97 following oral administration of 0.25 mg tritium labelled methylatropine in man.

plasma concentration. Following oral administration of 0.2 to 2.5 mg/kg, the concentration of radioactivity in the mandibular gland and heart is only 1-2 times the plasma concentration. The concentration in the gastric mucosa is about the same as that following subcutaneous injection.

The comparisons of plasma concentrations and heart rate are summarized in fig. 7 (The plasma concentration is calculated from the concentration of radioactivity in the plasma and the specific radioactivity of the methylatropine administered without correction for metabolites). It is seen that, compared with subcutaneous injection, a 25 times higher oral dose is needed to produce a comparable pharmacological effect (same increase in heart rate and pupil size).

Metabolic disposition of methylatropine in man

0.25 mg (125 μ Ci) methylatropine iodide was taken orally by one of the authors (body weight 70 kg) in the form of a solution in water. No pharmacological effects were noticed except for a feeling of dryness in the mouth. 25% of the dose was excreted in the urine within 24 hrs. Only 1.5% was recovered between 24-48 hrs. The major part (18%) was excreted within 4 hrs (fig. 3).

The radioactive components in an aliquot of urine collected between 85 and 140 min. after drug administration were separated on an Amberlite XE-97 resin as described earlier (20 ml of urine to 7 ml resin). The results are shown in fig. 8. As seen, more than 90% of the radioactivity in the

urine is in the form of metabolites. By the addition of labelled methylatropine to the urine, it could be verified that the unchanged drug was completely separated from this peak (fig. 8). It could not be established whether the 8% of radioactivity absorbed on the column and eluted with HCl was in the form of unchanged methylatropine or its metabolites. The amount of radioactivity excreted in the faeces within 72 hrs was 30% of the administered quantity.

Discussion

In all three species studied (mouse, dog and man) about 25% of the orally administered radioactivity is excreted in the urine within 24 hrs. The major part is excreted within 4 hrs indicating that the proximal part of the jejunum-ileum is the principal site of absorption. The results are in agreement with those obtained with isolated rat gut where 25% was absorbed during 3 hrs of perfusion (LEVINE 1959). Unfortunately it cannot be concluded with certainty whether the drug is absorbed in an unchanged state or not. It is evident that, following oral administration, two additional metabolites are present in the urine which are not present after parenteral administration. These two compounds appear to be identical with the products formed by hydrolysis of methylatropine and in mice comprise about 30% of the radioactivity in the urine. Thus it seems probable that a substantial hydrolysis occurs in the gastrointestinal tract. It is known that certain bacteria contain esterases which split atropine (BERENDS *et al* 1967). The degradation of the compound may not only be the result of the activity of bacterial enzymes since it has been shown that a closely related compound, hyoscine n-butylbromide, is about 5 times more toxic following instillation into the duodenum than following instillation into the stomach (WICK 1967).

In contrast to mouse and man, which excrete very little unchanged drug in the urine following oral administration, a substantial amount is also excreted unchanged by the dog following this route of administration.

The distribution of the drug is rather interesting and is very similar to that of atropine (GOSSELIN *et al* 1955; ALBANUS *et al* 1968a & b). Following parenteral administration to dogs, a considerable uptake occurs in the mandibular gland, the heart, the ciliary body and stomach. This probably explains the sensitivity of these tissues to the blocking effect of methylatropine, but it is not likely that the uptake is due to the presence of cholinergic receptors. This conclusion is based on the fact that choline acetyltransferase, a marker of cholinergic synapses, has been demonstrated

in the atria but not in the apex of the heart (HEBB 1956) whereas we found no difference in the uptake of radioactivity between these two parts of the heart.

The accumulation of radioactivity in the stomach is difficult to evaluate. About the same concentration ratio is found for all doses tested and after both routes of administration. The figures obtained following oral administration (20 times the plasma concentration) could be regarded as the result of inadequate washing were it not for the fact that the same percentage was accumulated following subcutaneous injection. The question is whether it is merely a coincidence that the figures are similar following both routes of administration or whether they are a result of absorption from the blood. The presence of relatively large amounts of radioactivity in the gastric contents following parenteral administration to mice and dogs indicate that radioactivity might be transported from the blood into the gastric lumen.

Following oral administration, the uptake in the other tissues studied is much less significant and occurs only with relatively high doses (1.0 and 2.5 mg/kg). This difference in uptake following the two routes of administration could be due to a high proportion of metabolites in the plasma (assuming that the unchanged drug is mainly accumulated) with the lower doses. In the case of atropine it has been shown that the unchanged drug is rather selectively accumulated in the salivary gland (ALBANUS *et al* 1968a).

In the dog experiments, an attempt was also made to investigate the relationship between the effect on the heart rate and the concentration of methylatropine in the plasma and heart (cf fig. 7). Following subcutaneous injection, the time course of the plasma on heart rate was similar to the time course of the plasma concentrations. The concentration in the heart is about 7 times higher than in the plasma. Although the percentage of metabolites in the plasma is not known, the error in the estimation of plasma and heart levels is probably not very great in the case of subcutaneous injection, since about 80% of the radioactivity in the urine is in form of unchanged methylatropine 4 hrs after injection. In the case of oral administration it is more questionable whether the figures presented are significant. However they indicate at least that compensatory mechanisms tend to counteract the effect of the drug. Following oral administration of 1.0 mg/kg the concentration of radioactivity in both the plasma and heart remain about the same for between 1 and 2 hrs. Despite the constant tissue levels, the heart rate falls markedly. The presence of compensatory mechanisms is also indicated by the fact that 0.1 mg/kg given orally (not shown in fig. 7) invariably produces a slight

bradycardia. This has also been observed in experiments in man (MÖLLER & ROSÉN 1967)

The general conclusion from the present study is that not only slow absorption but also extensive metabolic transformations explain the well-established fact that much higher oral than parenteral doses are needed to produce comparable pharmacological effects.

The high uptake in the stomach even after oral administration makes it possible that the drug may produce a local effect, without any noticeable effects on heart rate or salivary secretion.

Summary

The fate of tritium labelled methylatropine has been studied in mice, dogs and man. Following subcutaneous administration, about 50% of the radioactivity is excreted in the urine within 2 hrs. In mice and dogs, 40 and 80% (respectively) of the radioactivity in the urine is in the form of the unchanged drug.

Following oral administration 15 to 20% of the radioactivity is excreted in the urine within 6 hrs in the three species studied. In the mouse and man, less than 8% is in the form of unchanged methylatropine. Following the latter route of administration, two metabolites are found which are not present following subcutaneous administration. Chromatographic and electrophoretic evidence indicates that they are products formed by hydrolysis of methylatropine. It is believed that they are formed in the gastrointestinal tract.

In mice and dogs, a considerable uptake occurs in the salivary gland, ciliary body, heart and stomach following parenteral administration. On oral administration, the uptake in these organs occurs only after very high doses, except in the case of the stomach. In the dog, the oral doses needed to produce the same pharmacological effects are 25 times higher than with the subcutaneous doses.

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Histochemically Observable Alterations in Enzyme Pattern of Rat Myocardium, Caused by Parathion (E 605)

By

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(Received October 21 1968)

Parathion containing preparations, widely used in agriculture as highly potent insecticides have in many instance caused fatal poisoning of human subjects, either accidentally or intentionally (ALHA 1960). Parathion is an inhibitor of acetylcholine esterase. The accumulation of acetylcholine markedly disturbs neuromuscular transmission. Death following parathion poisoning has been attributed to asphyxia, partially due to bronchial constriction and pulmonary oedema, and partially due to paralysis of striated muscles and the respiratory centre (MÖLLER 1958). Since systemic anoxia aggravates the myocardial ischaemia in cases of coronary insufficiency (PLOTZ 1963), it can be assumed that during prolonged parathion poisoning the myocardial ischaemia will be severe enough to produce fresh myocardial infarction.

Enzyme histochemical methods are capable of revealing fresh myocardial anoxic lesions (JÄSKELÄINEN 1968) and hence, these methods have been used in the present study in order to demonstrate early alterations in the enzyme pattern of the rat myocardium following parathion poisoning.

Material and Methods

The experimental material consisted of 42 three months old albino rats with an average weight of 270 g (\pm 20 g). Parathion (preparation from Bayer AG) was given in each six animals i.e. 5 mg/kg, 20 mg/kg, 15 mg/kg, 12.5 mg/kg and 10 mg/kg respectively. Three animals in each group received 1 mg/kg atropine, injected subcutaneously after the onset of first signs of poisoning (slight twitching of muscles and erection of the whiskers). The dose was divided into three parts given at 5 minutes intervals.

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The following groups served as control material: a) three animals were strangled, causing increasing hypoxia for 10 minutes; b) three animals were killed by carbon monoxide; the poisoning caused death within 15-30 minutes; c) three animals received 1 mg/kg atropine in three doses without previous administration of parathion; d) three animals were killed by decapitation.

In each case the heart muscle was excised immediately after death. The specimens were frozen without delay in isopentane maintained at -70°C with a mixture of dry ice and acetone. The freezing period was approximately 10 seconds, after which 12 sections from each sample (20 μ) were cut with a rotary microtome at -20°C . The sections were further processed within a few minutes.

In the sections, phosphorylase activity was demonstrated by the method of T. KURIKI & KURIAKI (1955) as modified by TAKENAKI (1958). Cytochrome oxidase activity was visualized by the method of BURROUGHS (1959), using N,N'-para-amino-diphenylenediamine and 1- α -hydroxy naphthol acid. Succinate dehydrogenase activity was demonstrated by the method of NACHLAS, TSOU, DESOUZA, CHENGO & SELIGMAN (1957), using Nitro-BT as electron acceptor.

Results

The results are summarised in table 1. The study showed, that parathion poisoning caused definite diminution in the phosphorylase activity as

Table 1

Results of the study

Para- thion mg/kg	Atro- pine 1 mg/kg	Duration of toxic symptoms	Phosphor- ylase activity	Cytochrome oxidase activity	Succinic de- hydrogenase activity
25	-	7-10 min.	+	+++	+++
25	+	15-16 min.	++	+++	+++
20	-	10-17 min.	++	+++	+++
20	+	15-18 min.	+	+++	+++
15	-	15-20 min.	+-	+++	+++
15	+	(killed) 2 hr	+	+++	+++
12.5	-	20-190 min.	-	+++	+++
12.5	+	(killed) 2 hr	+	++	+++
10	-	(killed) 2 hr	++	+++	+++
10	+	(killed) 2 h	++	+++	+++
-	+	(killed) 15 min.	+++	++	+++
-	-	strangulation	-	+++	+++
-	-	CO-poison.	+	++	+++
-	-	control	+++	+++	+++

+++ = unchanged activity

++ = slight, patchy diminution in the activity

+

= clear decrease in the activity

- = no trace of enzyme activity



Fig. 1. Phosphorylase activity in the myocardium of the rat after parathion poisoning. Death occurred 7 minutes after the onset of toxic symptoms. The dose of parathion was 25 mg/kg. Magnification $\times 60$.

soon as 5-7 minutes after the onset of symptoms of poisoning. Initially the loss of enzyme activity was found in focal areas. Since the survival after parathion administration varied from 7-17 minutes, the extent of phosphorylase depletion from the myocardium was observed to be related to

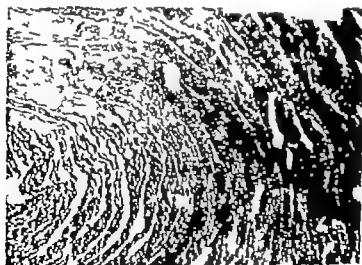


Fig. 2. Phosphorylase activity of the rat myocardium in parathion poisoning. Death occurred 18 minutes after the onset of toxic symptoms. The dose of parathion was 15 mg/kg. Magnification $\times 60$.



Fig. 3. Phosphorylase activity of the normal rat myocardium. No clear alterations in the enzyme activity are seen. Magnification $\times 60$.



Fig. 4. Phosphorylase activity of the myocardium after strangulation. Note the thin layer beneath the endocardium, which has retained its enzyme activity; the whole myocardium shows no trace of enzyme activity. Magnification $\times 60$.



Fig. 5 Phosphorylase activity in the myocardium after carbon monoxide poisoning. Death occurred after 15 minutes. Cf. fig. 1 and 2. Magnification $\times 60$.

the duration of the poisoning symptoms. This is clearly shown in the fig. 1 and 2. The diminution of phosphorylase activity however was not complete in the myocardium of parathion poisoned animals.

The processing of the samples, as described above, did not alter the phosphorylase activity of the myocardial cells. This is demonstrated in fig. 3 which shows the phosphorylase activity of the normal rat myocardium.

The administration of atropine did not prevent the observed change in phosphorylase activity of the myocardial cells. On the contrary the prolongation of the poisoning symptoms seemed to enhance the depletion of enzyme activity from the myocardium. In three cases the administration of atropine prevented death of the animals. In these instances the animals were killed 2 hours after the administration of parathion. No clear signs of altered enzyme activity were seen.

Since the periods of intoxication were quite short, varying from 7-17 minutes, the cytochrome oxidase and succinic dehydrogenase enzymes did not show any clear signs of diminished activity.

In the control material, strangulation caused total disappearance of

phosphorylase activity (fig. 4) from the myocardium during the 10 minutes period of anoxia. Carbon monoxide poisoning altered the phosphorylase activity to the same extent as parathion. After 15 minutes the myocardium showed moderate depletion of enzyme activity (fig. 5). In 30 minutes there was a considerable loss of phosphorylase activity. Cytochrome oxidase and succinic dehydrogenase activities did not alter during these periods of systemic anoxia.

In the myocardium of animals that had been decapitated or which had received atropine and then been decapitated, no signs of altered phosphorylase (fig. 3), cytochrome oxidase or succinic dehydrogenase activity could be detected.

Discussion

It has been pointed out that systemic shock or anoxia causes myocardial damage (MARTIN & HACKEL 1963, NANIKAWA, HAMAOKA & AWATA 1960) as can be seen by the demonstration of alterations in the enzyme pattern of the myocardium. These alterations appear after prolonged hypoxia, varying from 3½ to 7 hours (NANIKAWA, HAMAOKA & AWATA 1960) in the case of succinic dehydrogenase. Cytochrome oxidase has been observed to diminish after 2-3 hours of haemorrhagic shock (MARTIN & HACKEL 1963).

BLOOM (1956) showed, that anoxic work, done by the heart muscle, readily diminished the glycogen content of the myocardium. Since the phosphorylase activity diminution parallels that of the glycogen (KRUG 1967, JÄÄSKELÄINEN 1968) in the myocardial infarction, it is apparent, that very short periods of anoxia of the heart will deplete the myocardial phosphorylase activity.

In the present study the results showed that no clear alterations in the succinic dehydrogenase or in the cytochrome oxidase activity could be detected after parathion poisoning, when the toxic symptoms were of shorter duration than 17 minutes. This is in accordance with previous observations, which have shown, that, e.g. in myocardial infarction, the first signs of diminished enzyme activities are seen after a period of ischaemia of 1-2 hours (JÄÄSKELÄINEN 1968).

On the other hand the phosphorylase activity was observed to diminish after intoxication for 7 minutes. The degree of enzyme activity loss corresponded to the duration of the dyspnoea. Atropine did not prevent the loss of phosphorylase activity. On the contrary the prolongation of intoxication symptoms, caused by the administration of atropine enhanced the decrease in enzyme activity. In those cases in which the atropine alleviated the anoxic symptoms, no diminution in phosphorylase

activity of the myocardium was observed. This substantiated the observation that the depletion of phosphorylase activity in the myocardial cells is related to the duration of dyspnoea. It is interesting to note that the carbon monoxide poisoning, which brought about death within 15–30 minutes, caused a similar diminution of enzyme activity in the myocardium as did "moderate" parathion poisoning. By contrast, a maximal parathion dose caused a deep depletion of phosphorylase activity of the myocardium which partially corresponded to the observations on the effect of strangulation on myocardial phosphorylase activity.

In addition to the systemic anoxia, some local factors might be responsible for the patchy focal diminution of myocardial phosphorylase activity. Disturbances in the coronary circulation are often seen in severe anticholinesterase poisoning. Local reactions in the myocardial vascular bed may cause blockage of coronary circulation to some parts of the myocardium. This may explain the observed focal areas of diminished phosphorylase activity in the myocardium (cf. fig. 1). The loss of endogenous enzymes has been considered as one of the first signs of irreversible changes observed in dying myocardial cells (WARTMAN 1963). Thus, it is probable, that the observed focal areas represent the first stages of focal necroses i.e. myocardial infarctions. The deep myocardial anoxia, caused by parathion poisoning, leads to the depletion of intracellular enzymes, in the first place phosphorylase. However other intracellular enzymes (dehydrogenase and cytochrome oxidase) decrease in the later phases of anoxia. This has also been shown for myocardial infarction (JÄÄSKELÄINEN 1968).

The present study confirms previous investigations showing that atropine is an important therapeutic measure in parathion poisoning. In addition, in the treatment of parathion poisoning the measures taken against general anoxia are very important, particularly when there is evidence or suspicion of coronary heart disease.

Summary

The effect of parathion poisoning on myocardial enzymes was studied histochemically. The results showed that systemic anoxia, as well as any local reactions in the myocardial vascular bed caused by parathion poisoning, led to depletion of myocardial phosphorylase activity. This change corresponded to the duration of the anoxic symptoms. Injection of exceptionally massive doses of parathion, which caused death within a few minutes, caused a drastic depletion of myocardial phosphorylase activity. No clear signs of diminished cytochrome oxidase or succinic

dehydrogenase activity could be detected in the early phases of parathion poisoning. The results substantiate the concept that systemic anoxia during prolonged parathion poisoning may possibly give rise to fresh myocardial infarction, which is not totally prevented by the use of antidotes. This influences the treatment of parathion poisoning in cases with previous coronary heart disease.

Acknowledgements

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Oestrogen Binding in the Mouse Uterus 17 β -Oestradiol and meso-Hexaestrol Compete for the Binding Sites

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In addition to the natural oestrogens which are steroids, there are many synthetic non-steroidal oestrogens. From previous investigations it is known that some natural as well as synthetic oestrogens accumulate in target organs. For instance, the natural oestrogen 17 β -oestradiol is taken up by the reproductive tissues of the rat (e.g. JENSEN & JACOBSON 1962 AXELROD & EISENFELD 1965) and the mouse (STONE *et al* 1963 TERENTIUS 1965). The synthetic oestrogen meso-hexaestrol is similarly taken up by the reproductive tissues of the goat and sheep (GLASCOCK & HOEKSTRA 1959) the rat (JENSEN *et al* 1966) the ewe (ROBINSON 1965) and the mouse (TERENTIUS 1966b).

It is questionable whether natural and synthetic oestrogens are taken up by the same mechanisms and share common binding sites in the target organs. A number of experiments have been carried out *in vivo* in the rat (NOTESBOOM & GORSKI 1965) and in the mouse (TERENTIUS 1966a & b) as well as *in vitro* in the mouse (TERENTIUS 1966c) which show that the uterine uptake of 17 β -oestradiol can be inhibited by meso-hexaestrol and vice versa. From these experiments there is strong indirect evidence for believing that 17 β -oestradiol and meso-hexaestrol compete for common binding sites. However these earlier experiments were performed with one tritium-labelled oestrogen while the competitor was unlabelled. In the light of present-day knowledge of allo-steric effects it was felt that the problem had to be studied directly. When it was found possible to measure the concentrations of tritium-labelled 17 β -oestradiol and meso-hexaestrol separately (after a chromatographic separation) the present work was initiated. In addition, the interaction between 17 β -oestradiol and meso-

hexoestrol was studied in greater detail. The experiments were carried out *in vitro* in order to avoid any complications due to metabolism, turn-over etc.

Materials and Methods

Tritium-labelled 17β -oestradiol (oestra-1,3,5(10)-triene 3 17β -diol with tritium at the 6 and 7 positions) was obtained from New England Nuclear Corp., Boston Mass., U.S.A. Its specific activity was 145 $\mu\text{Ci}/\mu\text{g}$. Tritium-labelled meso-hexoestrol, specific activity 160 $\mu\text{Ci}/\mu\text{g}$, was the same as used in previous experiments (TERPSTRA 1966c). The radiochemical purity of the radioactive compounds was more than 95% as judged by thin layer chromatograms. Non-radioactive 17β -oestradiol, m.p. 173-4 was obtained from Sigma Chemical Co. St. Louis, Mo., U.S.A. while non-radioactive meso-hexoestrol, m.p. 186-7 was prepared by the author.

Female mice of the N.M.R.I. strain were used throughout. They were 14-17 days old and weighed 8-10 g at the time of the experiment.

The experimental procedure has been described in detail elsewhere (TERPSTRA 1968). The animals were killed by cervical dislocation and the uteri and occasionally the diaphragms were dissected out. Uteri were divided at the cervix into two equal parts and from the diaphragms, pieces weighing 3-4 mg were cut out. Two half uteri and occasionally two pieces of the diaphragm from each of two animals were incubated together in a flask. The incubation medium was Krebs-Ringer phosphate buffer pH 7.4 with 2% (w/v) bovine albumin. Each flask contained 3 ml of medium. The flasks were shaken in Warburg apparatus at constant temperature. Basically the tissues were first incubated with radioactive oestrogen 1:37 for 1 hour and then "washed" for a period of 1 hour at 25°C in a buffer with high concentration (0.1 $\mu\text{g}/\text{ml}$) of the appropriate non-radioactive oestrogen. The residual radioactivity in the tissues after washing is referred to as "bound" oestrogen.

After incubation the tissues were digested with hyamine 63 (Packard Co., La Grange Ill., U.S.A.) and prepared for liquid scintillation counting. Radioactivity was recorded on a Packard Tri-Carb 4 liquid scintillation spectrometer. The recorded counts were transformed into disintegrations per minute and unit wet weight (DPM/mg).

In a few experiments (fig. 4) simultaneous incubations with labelled 17β -oestradiol and labelled meso-hexoestrol were carried out. After incubation the uteri from each group were pooled, homogenized on ice and extracted with ethanol. 30 μg of each non-radioactive oestrogen was added as carrier. The ethanolic extract was then processed in an extraction procedure as described by TERPSTRA (1966b). Since the two oestrogens have very similar partition coefficients, they can be expected to be extracted with the same recoveries in the different steps. The phenolic fractions from the extraction contained practically all the radioactivity. They were subjected to chromatography on silica gel thin layers (Kieselgel H₂₅₄ Merck, Darmstadt, Germany) with chloroform:diethylamine 9:1 (v/v) as developer. After chromatography the spots with oestrogens were located in UV-light, and appropriate fractions of the thin layer from the whole developed length were scraped down into scintillation vials. 0.5 ml of methanol was added to elute the radioactivity from the silica gel. After standing for 1 hour 5 ml of scintillation solution was added and the radioactivity was measured. On all thin layer chromatograms examined, there was sharp separation between the two spots of radioactive oestrogen. More than 90% of the total radioactivity was located in the two oestrogen spots after chromatography.

The extent of binding of the oestrogens to albumin was studied by dialysis. Visking dialysis cassettes with 1.5 ml of 2% albumin solution in buffer were put into flasks with

6 ml of buffer containing either radioactive oestrogens. The flasks were incubated at 37 for two hours. From each flask duplicate samples of 200 μ l aliquotes from the dialysate and the solution were measured for tritium content.

In all of the figures and tables the amounts of the oestrogens are expressed on a weight basis. The molecular weights of 17 β -oestradiol and meso-hexaestrol, 272 and 270 respectively are so similar that comparisons on a molar basis are easy to perform.

Results

The quantities of the two oestrogens, 17 β -oestradiol and meso-hexaestrol which are bound to the albumin of the incubation medium under the experimental conditions described are comparable (table 1). meso-Hexaestrol was bound less strongly than 17 β -oestradiol, particularly at the highest concentration.

When half uteri from immature mice are incubated with each of the tritium-labelled oestrogens separately the uterus selectively takes up and retains the oestrogen in question. The time-course of uptake of 17 β -oestradiol and meso-hexaestrol by the uterus is shown in fig. 1. The concentrations used were far below saturation. It can be seen that from an incubation period of 30 min. and onwards, the uptake is almost constant both for 17 β -oestradiol and meso-hexaestrol. The level of 17 β -oestradiol taken up by the uterus is about 3-5 times higher than the level of meso-hexaestrol at all the incubation periods studied. For all the subsequent experiments the 1 hour incubation period with radioactive oestrogen was standard.

In fig. 2 the relation between the bath concentrations of the two oestrogens (used singly) and the amounts taken up by the uterus is illustrated. Strips of the non-target tissue, the diaphragm, were included

Table 1

Binding of 17 β -oestradiol and meso-hexaestrol to serum albumin. Dialysis bags with 2% bovine albumin were incubated for 2 hours at 37 °C with solutions of the oestrogens. Concentration ratios show the ranges of 3 separate experiments.

Oestrogen	Bath concentration of oestrogen before dialysis (μ g/ml)	After dialysis,
		Concentration inside bag Concentration outside bag
17 β -Oestradiol	0.0005	8.5-11.3
17 β -Oestradiol	0.05	8.8-10.8
meso-Hexaestrol	0.0005	6.0-8.5
meso-Hexaestrol	0.05	3.2-5.4

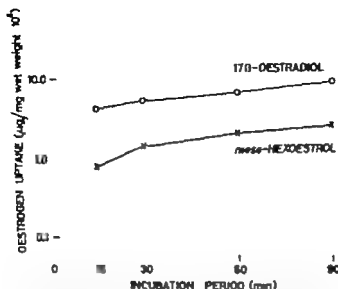


Fig. 1 Time-course of binding of 17 β -oestradiol and meso-hexaestrol in the uterus. The uteri were incubated for the indicated periods at 37° with 0.001 μ g/ml of tritium-labelled 17 β -oestradiol or meso-hexaestrol. The tissues were then transferred to other flasks with 0.1 μ g/ml of the appropriate unlabelled oestrogen and incubated at 22° for 1 hour. There were used from 6 animals per group.

in this experiment. The values of uterine uptake on the ordinate are corrected for the amount in the diaphragm: they therefore represent the additional uterine uptake. The uptake values for the highest concentrations are very uncertain, however, since they lie close to the saturation level of the uptake. This experiment, as well as the previous one, shows that 17 β -oestradiol is taken up more avidly than meso-hexaestrol by the uterus. The difference becomes even more distinct as the concentrations in the bath increase. It can also be seen that the maximum amount of 17 β -oestradiol taken up by the uterus during the 1 hour incubation periods is higher than that of meso-hexaestrol.

The preferential uptake of tritium labelled 17 β -oestradiol or meso-hexaestrol by the mouse uterus can be saturated (fig. 2). Fig. 3 shows how varying concentrations of non-radioactive 17 β -oestradiol and meso-hexaestrol affect the uptake of labelled 17 β -oestradiol. They both inhibit the uptake of labelled 17 β -oestradiol, meso-hexaestrol being about ten times less potent an inhibitor than 17 β -oestradiol. In sufficient amounts however it is capable of suppressing the binding of labelled 17 β -oestradiol completely.

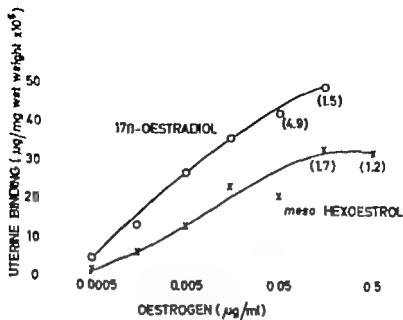


Fig. 2. Extent of uterine binding of 17β -oestradiol and meso-hexaestrol as function of the bath concentration. Uteri and pieces of the diaphragm were incubated for 1 hour at 37° with the indicated concentrations of radioactive oestrogen. The tissues were then incubated for 1 hour at 25° in other flasks with $0.1 \mu\text{g/ml}$ of the appropriate unlabelled oestrogen.

There were tissues from 6 animals per group. Values on the ord axis represent $\frac{\text{C uterus}}{\text{C diaphragm}}$ (C = concentration) of the oestrogens. Values in brackets are $\frac{\text{C uterus}}{\text{C diaphragm}}$.

In other experiments (TERENIUS 1968) it was found that high concentrations of non-radioactive 17β -oestradiol in the washing medium (cf Methods) reduce the binding of 17β -oestradiol in the uterus by some 30–40 per cent. A comparison between the capacity of 17β -oestradiol and meso-hexaestrol in the washing medium to affect the binding of labelled meso-hexaestrol revealed no differences (table 2).

The experiments in fig. 3 show that non-labelled meso-hexaestrol can block the uptake of 17β -oestradiol. In the following experiment half-uteri were incubated in varying mixtures of 17β -oestradiol and meso-hexaestrol, both labelled with tritium. The concentrations of the two oestrogens were estimated separately (see Methods). The incubation experiment revealed (fig. 4 exp 1) that increasing amounts of meso-hexaestrol reduce the uptake of 17β -oestradiol while the uptake of meso-hexaestrol simultaneously increases. The total extent of binding site occupation however becomes somewhat less with meso-hexaestrol. In exp 2 (fig. 4) a lower

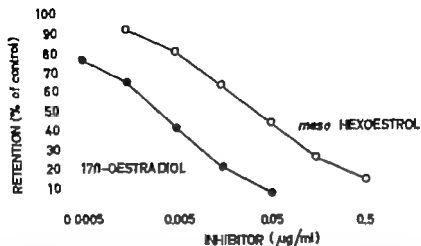


Fig. 3 Inhibitory effect of non-radioactive 17 β -oestradiol or meso-hexaestrol on the uptake of radioactive 17 β -oestradiol by the uterus. Uteri were incubated for 1 hour at 37 with 0.001 μ g/ml of tritium-labelled 17 β -oestradiol only (control) or together with the indicated concentrations of unlabelled 17 β -oestradiol or meso-hexaestrol. The uteri were then transferred to other flasks containing 0.1 μ g/ml of unlabelled 17 β -oestradiol and incubated for 1 hour at 25.

There were uteri from 4 animals per group.

concentration of 17 β -oestradiol was used, meso-Hexaestrol did not greatly affect this uptake in the concentrations studied but additional amounts of meso-hexaestrol were taken up. An increase in the concentration of 17 β -oestradiol lowered the amount of meso-hexaestrol taken up.

Table 2

Effect of non-radioactive 17 β -oestradiol or meso-hexaestrol added to washing medium on the retention of radioactive meso-hexaestrol by the uterus. Incubation for 1 hour at 37 with 0.001 μ g/ml tritium-labelled meso-hexaestrol was followed by washing for 1 hour at 37 in buffer with either 0.05 μ g/ml non-radioactive 17 β -oestradiol or 0.05 μ g/ml non-radioactive meso-hexaestrol. Uteri were taken from 9 animals. Mean values \pm standard error of the mean are given.

Oestrogen in washing medium	Content of radioactivity in the uterus relative to the medium
17 β -Oestradiol	2.09 \pm 0.07
meso-Hexaestrol	2.00 \pm 0.07

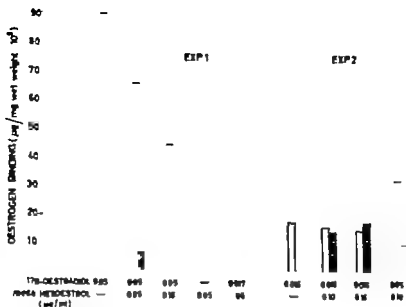


Fig. 4 Incubation of uteri in various mixtures of radioactive 17β -oestradiol and radioactive meso-hexaestrol. The incubations were performed as in fig. 3. After incubation the tissues were pooled (8 uteri per group), and the oestrogens extracted and chromatographed on silica gel thin layers. The spots on the chromatograms containing the oestrogens were eluted and their tritium content measured. Open columns in the figure represent the amount of 17β -oestradiol bound while hatched columns represent meso-hexaestrol.

Discussion

Direct chromatographic separation of 17β -oestradiol and meso-hexaestrol was only possible in basic solvents, while acid or neutral solvents gave little or no separation (cf STAHL 1962). A possible reason is that hexaestrol contains two phenolic groups while oestradiol only contains one. The phenolic hydrogen is slightly acid and partly dissociated in the basic solvent, which reduces the relative migration of meso-hexaestrol on the chromatograms.

Despite the fact that 17β -oestradiol is bound to the albumin of the medium to a greater extent than meso-hexaestrol (table 1), 17β -oestradiol is more avidly taken up by the uterus than meso-hexaestrol at the same concentrations (figs. 1 & 2). In the inhibition experiment too (fig. 3), the apparent affinity of 17β -oestradiol for the binding sites is at least ten times that for meso-hexaestrol. It is also possible that more 17β -oestradiol than meso-hexaestrol can be maximally taken up by the uterus (fig. 2). The incubations with mixtures of labelled 17β -oestradiol and labelled meso-hexaestrol (fig. 4) showed an interaction during uptake which was

at least in part, competitive. However when the uptake of 17β -oestradiol was suppressed by increasing concentrations of meso-hexaestrol, the suppressed 17β -oestradiol was not completely replaced by meso-hexaestrol. This might be an artifact, however due to washing out of accumulated meso-hexaestrol in the last incubation.

The oestrogen taken up by the uterus is released slowly on washing. The release is accelerated by the presence of non-radioactive oestrogen in the washing medium (TERENTUS 1968). Table 2 shows that non radioactive 17β -oestradiol and meso-hexaestrol are about equally active depleters of radioactive meso-hexaestrol. One might have expected from the observed differences in affinity during uptake that 17β -oestradiol would be more effective. Uptake and release however are apparently not reverse processes. TERENTUS (1967) suggested from experiments with metabolic inhibitors that uptake and retention depend on different mechanisms. JENSEN *et al* (1968) also observed that the uptake process was different from the binding process. These investigators studied protein fractions obtained from rat uterine homogenates and found that one extra nuclear protein component was responsible for the accumulation and that another protein component was responsible for the binding in the nuclei of the cells. Such a two-step mechanism is compatible with present experimental data.

In vivo the subcutaneous injection of $0.01 \mu\text{g}$ of meso-hexaestrol into the immature mouse gives about 2×10^{-2} / of the injected dose per mg wet uterus 2 hours after the injection. An equal dose of 17β -oestradiol gives only about 1.5 times this concentration in the uterus (TERENTUS 1965 & 1966b). Furthermore, meso-hexaestrol is about $\frac{1}{3}$ as active as 17β -oestradiol in blocking the uptake of 17β -oestradiol- ^3H *in vivo* (TERENTUS 1966a). meso-Hexaestrol is thus comparatively more active *in vivo* than *in vitro*. A possible reason for this anomaly could be a longer half life of meso-hexaestrol than of 17β -oestradiol in the circulation. This view is supported by experiments of JENSEN *et al* (1966) in the rat, where they actually found a slower plasma clearance of meso-hexaestrol than of 17β -oestradiol.

Summary

Surviving uteri and occasionally strips of the diaphragm were incubated *in vitro* with radioactive 17β -oestradiol and meso-hexaestrol, either singly or in varying mixtures. After this incubation the tissues were washed and the residual tissue radioactivity referred to as bound oestrogen.

When used singly 17β -oestradiol was bound more avidly than meso-hexaestrol by the uterus. When mixed 17β -oestradiol and meso-hexo-

estrol interacted for the binding sites in a manner which at least in part was competitive meso-Hexoestrol however inhibited the uptake of 17 β -oestradiol without completely restoring the empty binding sites.

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Concentrations of Amitriptyline and Its Metabolites in Urine, Blood and Tissue in Fatal Amitriptyline Poisoning

By

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(Received September 19 1968)

Death from poisoning by amitriptyline (Saroten® 5-(3-dimethylaminopropylidene)-10,11-dihydrogen 5H-dibenzo-[a,d]-cycloheptene) is not a rare occurrence in Denmark. During the past year no less than five or six deaths due solely or mainly to the effect of amitriptyline were seen at this Institute. Very few publications deal with postmortem concentrations of amitriptyline in the blood and tissues. In one case of fatal amitriptyline poisoning (136 mg per kg body weight taken orally) SUNSHINE & YAFFE (1963) found a blood amitriptyline level of 2 µg/ml. McBAY (1967), in two fatal cases, found concentrations of 2 µg/ml and 11 µg/ml blood, respectively. In neither study was isolation and identification of the metabolites of amitriptyline attempted.

This paper reports the results of analyses in six cases of fatal amitriptyline poisoning, in which amitriptyline and three or four of its metabolites in the blood, urine and tissue were quantitatively determined.

Technique

3 ml of blood, liver homogenate or urine is extracted by shaking with 10 ml of 1 M sodium carbonate and 0.5 ml of 1,2-dichloroethane for 20 minutes (HERMANN & PULVER 1960). 15 ml of the dichloroethane phase is evaporated to dryness and the residue is thin-layer chromatographed (silica gel PF₂₅₄, Merck, activated at 110° for 30 minutes). Removal of contaminants is achieved by first developing the plate with a mixture of chloroform and ether 1:1. A large portion of contaminants will then settle at the solvent front. The plate is air dried and the actual chromatographic assay is performed with suitable solvent, cf. table 1.

Table 1

Approximate R_f -values (relative values in brackets) of amitriptyline and its metabolites.

Solvent system No.	1	2	3	4	5
Amitriptyline	0.56 (1.00)	0.81 (1.00)	0.85 (1.00)	0.86 (1.00)	0.40 (1.00)
Nortriptyline	0.28 (0.50)	0.72 (0.89)	0.61 (0.79)	0.57 (0.73)	0.62 (1.55)
Metabolite a	0.16 (0.29)	0.58 (0.79)	0.23 (0.27)	0.30 (0.35)	0.31 (0.78)
- b	0.71 (0.20)	0.12 (0.15)	0.08 (0.09)	0.09 (0.10)	0.50 (1.25)
- c	0.05 (0.09)	0.03 (0.04)	0 (0)	0 (0)	0.17 (0.42)

Solvent system No. 1: Chloroform, ether methanol: 85 + 15 + 20, followed by chloroform, ether methanol, conc. ammonia: 85 + 15 + 20 + 0.5.

Solvent system No. 2: Chloroform, acetone, diethylamine: 50 + 40 + 10.

Solvent system No. 3: Cyclohexane, chloroform, diethylamine: 70 + 20 + 10.

Solvent system No. 4: Benzene, diazane, acetone, conc. ammonia: 50 + 20 + 20 + 2.

Solvent system No. 5: Butanol, acetic acid, water: 80 + 20 + 20.

Visible spots are marked under ultraviolet light and measured spectrophotometrically after elution with methanol. The concentration is determined from the absorption value at λ_{39} m μ , the wavelength at which amitriptyline, nortriptyline and the other metabolites detected in this study reach their maximum, cf. fig. 2.

Results and Discussion

The results of plate chromatography are presented in table 1 where amitriptyline, the metabolite nortriptyline and the metabolites designated a, b and c are characterized by their R_f values in five different solvent systems. Extracts of urine from Case No. F 3/68 and extracts of liver and urine from Case No. D 272/67 were used.

Table 2 shows the results of blood, liver and urine analyses in six cases of fatal amitriptyline poisoning. It will be seen that the amounts present in the blood were fairly small in relation to the amounts ingested much higher concentrations of amitriptyline and nortriptyline were found in the liver. The metabolites designated a, b and c appeared mainly in the urine.

Case Records

The details of the six deaths from amitriptyline poisoning were as follows:

Case No. D 272/67 A woman aged 23 weighing 67 kg, was found unconscious. She had previously attempted suicide with amitriptyline.

Table 2

The concentrations of amitriptyline and its metabolites in blood and urine ($\mu\text{g}/\text{ml}$) and liver ($\mu\text{g}/\text{g}$) in fatal cases of amitriptyline poisoning.

Case No.	Estimated dose, orally	Material	Amitri- ptyline	Nortri- ptyline	Metabolites		Total
					b	a	
M 272/67	37 mg/kg	Blood	6	5	4	—	15
		Liver	72	98	18	—	188
		Urine	6	10	34	traces	49
D 284/67	46 mg/kg	Blood	18	—	—	—	18
		Liver	66	24	traces	—	90
		Urine	28	12	11	—	51
F 3/68	90 (?) mg/kg + barbiturate	Blood	3	2	—	—	5
		Liver	58	60	traces	—	118
		Urine	7	7	12	7	30
F 55/68	45 (?) mg/kg	Blood	5	—	—	—	5
		Urine	20	19	37	30	137
A 12573/67	36 (?) mg/kg + alcohol	Blood	9	9	—	—	18
		Liver	4	—	—	—	6
F 133/68	22 mg/kg + alcohol	Blood	59	31	5	—	95
		Liver	—	—	—	—	—

Total concentrations of amitriptyline and metabolites. All concentrations are calculated as amitriptyline hydrochloride.

She died 24 hours after hospitalization. In her bag were found a half empty bottle with Tegretol ® (carbamazepine NFN) tablets and an empty Saroten ® bottle (100 tablets each 25 mg) which had been handed out to her a few hours previously.

Autopsy revealed the presence of tablet fragments in the stomach contents. Barbituric acid, meprobamate and alcohol were not demonstrable in the blood samples.

Case No. D 284/67 A woman aged 35 weighing 54 kg, was found dead. A half empty bottle with Truxal ® (chlorprotixene NFN) tablets, a bottle with Noludar ® (methypirylone NFN) tablets and an empty Saroten ® bottle (100 tablets each 25 mg) were found in her bag.

Autopsy revealed the presence of tablet fragments in the stomach contents. Chlorprotixene, barbituric acid, meprobamate and alcohol were not demonstrable in the blood and urine.

Case No. F 3/68 A woman aged 43 weighing 55 kg, was found dead. Two empty Saroten ® bottles (200 tablets each 25 mg) and an empty 50 tablets allypropymal bottle were found at her bedside. A half empty bottle with Restenil ® (meprobamate NFN) tablets, a half empty bottle with pethidine tablets, an empty box which had contained 5 x 1 ml amp. Ketogan ® (5 mg ketobemidone + 25 mg 3-dimethylamino-1,1-diphenyl-butene-1,hydrochloride) and partially emptied antazolin, Mogadon ® (nitrazepam NFN) and Noludar ® (methypirylone NFN) bottles were found on further search.

Autopsy revealed the presence of tablet fragments in the stomach contents. Allypropymal was found in the blood and liver in concentrations of 72 µg/ml and 112 µg/g, respectively. Meprobamate and alcohol were not demonstrable.

Case No. F 55/68 A woman aged 48 weighing 55 kg, was found dead. No tablets were found nearby but an empty 100 tablets Saroten ® bottle and an empty 50 tablets Mekvalon ® (methaqualone NFN) bottle were found in her home.

Autopsy revealed the presence of tablet fragments in stomach contents. Methaqualone, meprobamate, barbituric acid and alcohol were not demonstrable in the blood samples.

Case No. A 12573/67 A man aged 24 estimated weight 70 kg, was found dead. An empty Saroten ® bottle (100 tablets each 25 mg) was found in the room.

Autopsy was not performed. The blood alcohol level was 0.76 per mille. Barbituric acid and meprobamate were not demonstrable.

Case No. F 133/68 A man aged 52, weighing 57 kg, was found dead. An empty Saroten ® bottle (50 tablets each 25 mg) and a partially emptied Stesolid ® (diazepam NFN) bottle were found in his home.

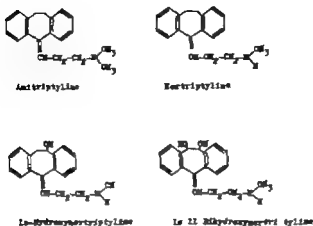


Fig. 1 Suggested metabolites of amitriptyline and nortriptyline.

Autopsy revealed the possible presence of tablet fragments in stomach contents. The blood alcohol level was 0.64 per mille. Barbituric acid, meprobamate and diazepam were not demonstrable.

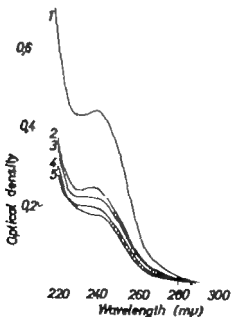


Fig. 2. Ultraviolet spectra of amitriptyline and its metabolites. 1 amitriptyline. 2 nortriptyline. 3 metabolite c. 4 metabolite a. 5 metabolite b.

Identification of Metabolites

It is known from the publications of HUCKER (1962) and others, that amitriptyline is demethylated in the body to nortriptyline. The present study confirmed that nortriptyline is the major metabolite. It was identifiable by plate chromatography in five different solvents. The identity of metabolites a, b and c could not be established. The results suggest, however, that they are hydroxylated metabolites of amitriptyline and nortriptyline, cf. the studies of metabolic degradation of nortriptyline by McMAHON *et al* (1963) and AMUNDSON & MANTHEY (1966) fig. 1.

It will be seen from the results recorded in table 1 that metabolites a, b and c are much more hydropophilic (solvent systems 1, 2, 3 and 4) than the parent compound and that the R_f value of metabolite b like that of nortriptyline (acid solvent system 5) is substantially increased. This suggests that metabolite b is a hydroxylated metabolite of nortriptyline, while metabolites a and c would seem to be corresponding hydroxylated metabolites of amitriptyline. The ultraviolet spectra in fig. 2 show that all the metabolites closely resemble amitriptyline.

Summary

Results of analyses in six cases of fatal amitriptyline poisoning are reported. The analytical method consists in extraction with 1,2-dichloroethane, plate chromatography of the extract, followed by elution and spectrophotometric measurement. The plate chromatographic properties of four metabolites of amitriptyline are reported and suggestions made as to their chemical composition.

Acknowledgement

Our thanks are due to H. Lundbeck & Co. Inc., Copenhagen, for supplying the reference substances amitriptyline and nortriptyline.

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Interaction of DOPA Decarboxylase Inhibitors with the Effect of α Methyl-dopa on Blood Pressure and Tissue Monoamines in Rats¹

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Several attempts have been made to explain the antihypertensive properties of α methyl-dopa (α MD) and the effects of this drug on monoamine metabolism. It is well known that α -MD can be metabolized *in vivo* to form α -methyl-dopamine (α -methyl-DA) and α -methylnoradrenaline (α -methyl-NA) (CARLSSON & LINDQVIST 1962 MUSCHOLL & MAHRE 1963 SCHÜMANN & GROBECKER 1964 TORCHIANA *et al* 1964 PORTER *et al* 1965) DAY & RAND (1964) obtained a moderate reduction of the response to stimulation of the peripheral sympathetic nerves after α MD and found α -methyl-NA to be a weaker vasoconstrictor than NA. The suggestion was made that α -methyl-NA formed from α MD may serve as a "false transmitter" in place of NA in the sympathetic nerves the effectiveness of sympathetic impulses would then be diminished, thus leading to a reduction in blood pressure.

A prerequisite of this hypothesis is that the prevention of the synthesis of the false transmitter from α -MD should abolish the hypotensive action. Only few studies seem to have been performed to determine the validity of this implication. SJÖERDGAARD *et al.* (1963) reported that an inhibitor of DOPA decarboxylase, α -hydrazino- α -methyl- β -(3,4-dihydroxyphenyl)-propionic acid (MK 485) was unable to counteract the hypotensive effect of α MD in man. However DAVIS *et al* (1963) found that in the rat the decarboxylase inhibitor N 2-hydroxybenzyl-N-methylhydrazine (NSD 1039) antagonized this effect.

¹These results were presented in preliminary form at the joint meeting of the British and Scandinavian Pharmacological Societies, Edinburgh, July 9th-11th, 1968 (HENNING 1968).

In view of these divergent results, the problem has been reinvestigated, using two different inhibitors of DOPA decarboxylase with apparently somewhat different properties, i.e. scryl-2,3 4-trihydroxybenzyl-hydrazine (Ro 4-4602) and the previously mentioned MK 485. The former inhibitor when given in sufficiently large doses, inhibits both peripheral and central decarboxylase (PLETSCHER & GEY 1963, BURKHARD *et al* 1962 & 1964), whereas MK 485 does not readily penetrate the blood brain barrier (PORTER *et al* 1962). Of these two inhibitors, only Ro 4-4602 was found to antagonize the hypotensive effect of α MD.

Methods

For the blood pressure experiments, male Sprague-Dawley rats weighing 180–250 g with renal hypertension were used. The mean arterial blood pressure was recorded in conscious, unrestrained animals through in-dwelling arterial catheters (Porovic & Porovic 1960). For technical details, see HANSSON (1967).

In the biochemical studies, normotensive rats of corresponding body weight were used. NA was determined by the method of BEATLER *et al*. (1958), DA and α methyl-DA as described by CARLSSON & LINQVIST (1962) and 3-hydroxytryptamine (3-HT) according to ANDÉN & MACHUSSON (1967). The drugs used were L- α -methyl-3 4-dihydroxyphenylalanine (α -methyldopa), scryl-2,3 4-trihydroxybenzyl-hydrazine-HCl (Ro 4-4602, calculated as the salt) and α -hydrazino- α -methyl β -(3 4-dihydroxyphenyl)-propionic acid, dissolved in 0.9% saline and injected i.p. Doses and intervals are given in the Results. Tests of significance were conducted with Student's *t*-test and analysis of variance (Dunn 1949) and *P* values equal to or less than 0.05 were regarded as significant.

Results

1 Ro 4-4602 series

A significant fall in mean arterial blood pressure was observed 1–12 hours after α MD 200 mg/kg, the maximum being reached after 3–6 hours (fig. 1). Return to control blood pressure occurred after 24 hours. Another group of rats were given three doses of the decarboxylase inhibitor Ro 4-4602 (200 mg/kg) 30 min. before, 1½ and 3½ hours after α MD 200 mg/kg. This pretreatment prevented the fall in blood pressure 1–6 hours after α MD administration (fig. 1). The difference between the changes in blood pressure in the two groups was significant with $P < 0.005$ after 1 hour and $P < 0.001$ after 3 and 6 hours. In these experiments however there was a delayed hypotensive effect 12–48 hours after α -MD ($P < 0.05$). In a third series of experiments Ro 4-4602 was given alone in the same dose schedule. This resulted in a similar response as that after α -MD and Ro 4-4602, i.e. a significant decrease in blood pressure was seen 12–48 hours after the first dose of the inhibitor. There was no significant difference between the decreases in the last two series.

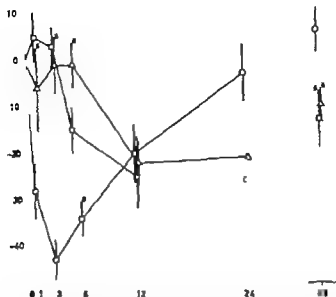


Fig. 1 *Oral* Changes in mean arterial blood pressure (mm Hg) of conscious renal hypertensive rats after the following treatments: α -methyl dopa 200 mg/kg i.p. (circles); the decarboxylase inhibitor Ro 4-4602, three doses of 200 mg/kg i.p. given $\frac{1}{2}$ hour before and $1\frac{1}{2}$ and $3\frac{1}{2}$ hours after α -methyl dopa 200 mg/kg p. (triangles); Ro 4-4602 alone as indicated above (squares). *Abscissa*: Time in hours. *Injection* at time zero. Basal blood pressure levels in the three groups were 162 mm Hg (S.E.M. = 8.3, $n = 8$), 159 mm Hg (S.E.M. = 6.8, $n = 6$) and 145 mm Hg (S.E.M. = 9.6, $n = 6$), respectively.

Table 1 gives the results of these procedures on the levels of tissue monoamines. The dose schedule was the same as in the blood pressure experiments except that only two doses of Ro 4-4602 were given to animals sacrificed three hours after α -MD. The findings may be divided into three parts:

- (1) α -MD alone significantly lowered NA in the heart and NA, DA and 5-HT in the brain after 3 and 6 hours. No effect was observed on femoral muscle NA.
- (2) Ro 4-4602 alone decreased the 5-HT content in the brain after 3 and 6 hours. Brain, heart and femoral muscle NA levels were reduced only after 3 hours. In contrast to the other amines, brain DA levels increased significantly in comparison to the control levels at both intervals studied.
- (3) Ro 4-4602 and α -MD in combination caused the same depletion of brain NA and 5-HT as α -MD alone after 3 and 6 hours. The effect seemed to be greater than that of Ro 4-4602 alone in the case of 5-HT after 3 hours and of NA after 6 hours. The levels of brain DA were the same as in the control animals and significantly higher than after α -MD alone.

Table 1

Levels of noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (5-HT) and α -methyldopamine (MDA) in the brain, NA and MDA in the heart and NA in the femoral muscle of rats treated as indicated in the left column. The values are means in $\mu\text{g/g}$ of the base number of experiments is indicated in brackets. P values were obtained by analysis of variance.

Treatment (Dose, time before sacrifice)	Brain				Heart		Femoral muscle
	NA	DA	5-HT	MDA	NA	MDA	NA
A. None	0.40 (7)	0.53 (4)	0.36 (4)	-	1.01 (7)	-	0.09 (7)
B. α -methyldopa 200 mg/kg (3 hr)	0.19 (3)	0.31 (3)	0.12 (3)	0.53 (3)	0.67 (3)	0.13 (3)	0.09 (3)
C. α -methyldopa 200 mg/kg (6 hr)	0.13 (3)	0.25 (3)	0.22 (3)	0.52 (6)	0.59 (3)	0.09 (3)	0.09 (3)
D. Ro 4-4602 (200 mg/kg, 3½ hr) + α -methyldopa (200 mg/kg, 3 hr) + Ro 4-4602 (200 mg/kg, 1½ hr)	0.19 (3)	0.53 (3)	0.17 (3)	0.15 (3)	0.65 (3)	0.00 (3)	0.06 (3)
E. Ro 4-4602 (200 mg/kg, 6½ hr) + α -methyldopa (200 mg/kg, 6 hr) + Ro 4-4602 (200 mg/kg, 4½ hr) + Ro 4-4602 (200 mg/kg, 3 hr)	0.13 (3)	0.43 (3)	0.19 (3)	0.22 (3)	1.20 (3)	0.03 (3)	0.10 (3)
F. Ro 4-4602 (200 mg/kg, 3½ hr) + 0.9% N Cl (3 hr) + Ro 4-4602 (200 mg/kg, 1½ hr)	0.24 (3)	0.78 (3)	0.90 (3)	0.01 (3)	0.71 (3)	0.01 (3)	0.05 (3)
G. Ro 4-4602 (200 mg/kg, 6½ hr) + 0.9% N Cl (6 h) + Ro 4-4602 (200 mg/kg, 4½ hr) + Ro 4-4602 (200 mg/kg, 3 hr)	0.34 (3)	0.66 (3)	0.25 (3)	0.01 (3)	0.99 (3)	0.00 (3)	- (3)
Variance within groups A-G	0.0032	0.0026	0.0010	0.0151	0.0378	0.0002	0.0001
P (n.s. = not significant)							
A-B	< 0.1	< 0.1	< 0.1		< 2.5		n.s.
A-C	< 0.1	< 0.1	< 0.1	-	< 1	-	n.s.
A-D	< 0.1	n.s.	< 0.1		< 2.5	-	< 0.1
A-E	< 0.1	< 2.5	< 0.1	-	n.s.		n.s.
A-F	< 0.1	< 0.1	< 5		< 5		< 0.1
A-G	n.s.	< 1	< 0.1		n.s.	-	
B-C	n.s.	n.s.	< 0.1	n.s.	n.s.	< 1	n.s.
B-D	n.s.	< 0.1	< 5	< 0.5	n.s.	< 0.1	< 0.1
B-E	n.s.	< 1	< 1	< 1	< 1	< 0.1	n.s.
B-F	n.s.	< 0.1	< 0.1	< 0.1	n.s.	< 0.1	< 0.1
B-G	< 1	< 0.1	< 0.1	< 0.1	n.s.	< 0.1	-

C-D	n.s.	< 0.1	n.s.	< 0.1	n.s.	< 0.1	< 0.1
C-E	n.s.	< 0.1	n.s.	< 0.5	< 0.5	< 0.1	n.s.
C-F	< 5	< 0.1	< 0.5	< 0.1	n.s.	< 0.1	< 0.1
C-G	< 1	< 0.1	n.s.	< 0.1	< 2.5	< 0.1	-
D-E	n.s.	< 2.5	n.s.	n.s.	< 0.5	n.s.	< 0.1
D-F	n.s.	< 0.1	< 0.1	n.s.	n.s.	n.s.	n.s.
D-G	< 1	< 0.1	< 2.5	n.s.	< 5	n.s.	-
E-F	< 5	< 0.1	< 0.1	n.s.	< 1	n.s.	< 0.1
E-G	< 0.1	< 0.1	n.s.	n.s.	n.s.	n.s.	-
F-G	< 5	< 2.5	< 1	n.s.	n.s.	n.s.	-

but lower than after Ro 4-4602 alone. Heart NA was decreased to the same extent as by either drug alone though only after 3 hours normal values were obtained after 6 hours. The same was true of femoral muscle NA. The accumulation of α -methyl DA in the brain and in the heart was significantly reduced at both intervals studied

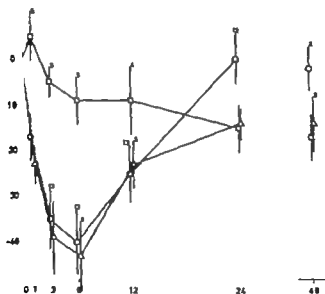


Fig. 2. *Oral*. Changes in mean arterial blood pressure (mm Hg) of conscious renal hypertensive rats after the following treatments: α -methyl dopa 100 mg/kg i.p. (circles); the decarboxylase inhibitor MK 485, four doses of 100 mg/kg i.p. $\frac{1}{2}$ hour before, and 1 $\frac{1}{2}$, 3 $\frac{1}{2}$ and 5 $\frac{1}{2}$ hours after α -methyl dopa 100 mg/kg i.p. (triangles); MK 485 alone as indicated above (squares). *Abscissa*: Time in hours. *Injectious* at time zero. Basal blood pressure levels in the three groups were 168 mm Hg (S.E.M. = 5.8, n = 14), 162 mm Hg (S.E.M. = 7.0, n = 8) and 155 mm Hg (S.E.M. = 10.4, n = 5), respectively.

Table 2

Levels of noradrenaline (NA), dopamine (DA) and α -methyldopamine (MDA) in the brain, NA and MDA in the heart and NA in the femoral muscle of rats treated as indicated in the left column. The values are means in $\mu\text{g/g}$ of the base number of experiments is indicated in brackets. F values were obtained by analysis of variance.

Treatment (Dose, time before sacrifice)	Brain			Heart		Femoral muscle
	NA	DA	MDA	NA	MDA	NA
A. None	0.40 (7)	0.53 (4)	-	1.01 (7)	-	0.09 (7)
B. α -methyldopa 100 mg/kg (3 hr)	0.15 (3)	0.42 (3)	0.45 (3)	0.57 (3)	0.16 (3)	0.09 (2)
C. α -methyldopa 100 mg/kg (6 hr)	0.12 (6)	0.49 (3)	0.30 (6)	0.66 (7)	0.14 (4)	0.16 (7)
D. MK 485 (100 mg/kg, 3½ hr) + α -methyldopa (100 mg/kg, 3 hr) + MK 485 (100 mg/kg, 1½ hr)	0.21 (3)	0.42 (3)	0.36 (3)	0.95 (3)	0.00 (3)	
E. MK 485 (100 mg/kg, 6½ hr) + α -methyldopa (100 mg/kg, 6 hr) + MK 485 (100 mg/kg, 4½ hr) + MK 485 (100 mg/kg, 3 hr) + MK 485 (100 mg/kg, 1½ hr)	0.12 (4)	0.39 (2)	0.42 (4)	0.94 (4)	0.00 (5)	0.09 (2)
F. MK 485 (100 mg/kg, 3½ hr) + 0.9% NCl (3 hr) + MK 485 (100 mg/kg, 1½ h)	0.61 (2)	0.68 (2)	0.00 (2)	0.93 (2)	0.00 (2)	-
G. MK 485 (100 mg/kg, 6½ h) + 0.9% NaCl (6 h) + MK 485 (100 mg/kg, 4½ h) + MK 485 (100 mg/kg, 3 hr) + MK 485 (100 mg/kg, 1½ h)	0.42 (4)	0.60 (3)	0.00 (3)	0.88 (4)	0.00 (3)	0.09 (2)
Variance within groups A-G	0.0032	0.0069	0.0090	0.0309	0.0011	0.0003
P(2)(n.s. = not significant)						
A-B	< 0.1	n.s.	-	< 0.1	-	No
A-C	< 0.1	n.s.	-	< 0.5	-	signif-
A-D	< 0.1	n.s.	-	n.s.	-	cance
A-E	< 0.1	< 5	-	n	-	between
A-F	0.1	< 5	-	n.s.	-	groups
A-G	n.s.	n.s.	-	n.s.	-	A-G
B-C	n.s.	n.s.	< 5	n.s.	n.s.	
B-D	n.s.	n.s.	n.s.	< 5	< 0.1	
B-E	n.s.	n.s.	n.s.	< 2.5	< 0.1	
B-F	< 0.1	< 0.5	< 0.1	< 5	< 0.1	
B-G	< 0.1	< 2.5	< 0.1	< 5	< 0.1	
C-D	< 2.5	n.s.	n.s.	< 5	< 0.1	
C-E	n.s.	n.s.	n.s.	< 2.5	< 0.1	
C-F	< 0.1	< 2.5	< 0.5	n.s.	< 0.1	
C-G	< 0.1	n.s.	< 0.5	n.s.	< 0.1	

D-E	< 5	n.s.	n.s.	n.s.	n.s.
D-F	< 0.1	< 0.5	< 0.1	n.s.	n.s.
D-G	< 0.1	< 2.5	< 0.1	n.s.	n.s.
E-F	< 0.1	< 0.5	< 0.1	n.s.	n.s.
E-G	< 0.1	< 1	< 0.1	n.s.	n.s.
F-G	< 0.1	n.s.	n.s.	n.s.	n.s.

2 MK 485 series

The results obtained with this decarboxylase inhibitor are shown in fig. 2 and table 2. MK 485 was given in four doses of 100 mg/kg 30 min before, as well as 1½, 3½ and 5½ hours after an injection of α -MD 100 mg/kg. Both α -MD alone and in combination with MK 485 produced a significant lowering of the blood pressure after 1-6 hours which was almost identical in the two series, i.e. the hypotensive response to α -MD was not blocked by the inhibitor. Similar to Ro 4-4602, MK 485 in combination with α -MD resulted in a slowly developing fall in blood pressure. The difference from α -MD alone was significant after 12 and 24 hours ($P < 0.005$ and $P < 0.01$ respectively). MK 485 alone also appeared to lower the blood pressure at late intervals ($P < 0.05$ after 48 hours) the changes however were not significantly different from those found after the inhibitor in combination with α -MD.

Tissue monoamine levels after the various treatments are given in table 2. Doses and times were the same as in the blood pressure experiments except that only two doses of Mk. 485 were given for the 3 hour values.

- (1) α -MD alone decreased the concentration of NA in the brain and in the heart but this low dose of α -MD did not alter brain DA levels. No effect was observed on femoral muscle NA levels.
- (2) MK 485 alone gave a significant increase in brain NA and DA after 3 hours. No change was observed in the NA levels in the heart or femoral muscle.
- (3) MK 485 and α -MD in combination lowered the brain NA to the same extent after 3 and 6 hours as α -MD alone. The brain DA levels were within control levels but significantly lower than after MK 485 alone. No depletion of heart NA was observed the values after 3 and 6 hours were significantly greater than those observed after α -MD alone. The accumulation of α -methyl-DA was almost completely abolished by MK 485 in the heart but unchanged in the brain at both intervals.

Discussion

In agreement with several other investigators (for references see MUSCHOLL 1966 HOLTZ & PALM 1966) it was found that the administration of α MD lowered the levels of catecholamines and 5-HT in various tissues. As first suggested by CARLSSON & LINDQVIST (1962) and confirmed in many subsequent studies (review by MUSCHOLL 1966) the depletion of NA and DA after α MD occurs mainly through a stoichiometric displacement of these amines by their corresponding α -methylated analogues. Inhibition of synthesis probably contributes to the effect, particularly in the case of 5-HT (SHARMAN & SMITH 1962 ROOS & WERDINUS 1963 PLETSCHER *et al* 1964a & b).

Administration of decarboxylase inhibitors resulted in complex changes in tissue monoamine levels. Ro 4-4602 moderately decreased the concentration of NA and 5-HT in the brain and of NA in the heart. Similar results have been reported previously the effect generally being attributed inhibition of the synthesis at the decarboxylation stage (BURKARD *et al* 1962 PLETSCHER & GEY 1963 PLETSCHER *et al* 1964a & b JOHNSON & PRITZLER 1966 ANDÉN *et al* 1967). However in the present study Ro 4-4602 significantly increased the brain DA levels. This is in contrast to the results of PLETSCHER *et al* (1964a) and is not readily explained in terms of decarboxylase inhibition. The simultaneous decrease in NA and increase in DA is suggestive of β -hydroxylase inhibition. Ro 4-4602 is in fact a weak inhibitor of this enzyme *in vitro* (BURKARD *et al* 1964). On the other hand, a significant inhibition of brain decarboxylase probably operates at the same time, causing a decrease in 5-HT and a marked reduction in the accumulation of α -methyl-DA after α -MD. The other decarboxylase inhibitor MK 485 significantly raised the concentrations of both NA and DA in brain tissue. Since MK 485 is a potent inhibitor of peripheral decarboxylase but seems to penetrate poorly into the brain (PORTER *et al* 1962), it is possible that these changes result from the increased availability of DOPA to the brain *via* the blood stream. Normally DOPA is not demonstrable in tissues or circulating blood but it has been found in tumours of nervous origin (WEIL MALHERBE 1956) and in the urine of patients with such tumours (STUDNITZ 1960). It is possible that the increased levels of brain DA after Ro 4-4602 may be explained at least in part in the same way as after MK 485 since recent observations indicate that, in small doses, the former drug behaves like MK 485 acting preferentially on peripheral decarboxylase (BARTHOLOMI & PLETSCHER 1968).

The interpretation of alterations in tissue monoamines after α -MD and decarboxylase inhibitors in combination is difficult in view of the varying

changes of these amines brought about by the latter drugs alone. Thus, Ro 4-4602 appeared to prevent the depletion of brain DA and heart NA by a MD but failed to influence the lowering of brain NA. On the other hand, a similar pattern of changes in brain amines was observed after the decarboxylase inhibitor alone. After MK 485 and a MD brain DA levels were significantly lower than after the former drug alone. This may indicate a lack of protection from the depleting action of a MD by the inhibitor. It should be emphasized, however, that both inhibitors completely prevented the formation of a methyl-DA in the heart after a MD while that in the brain was significantly reduced by Ro 4-4602 only. Although the concentrations of a methyl NA were not determined in these experiments, it appears most likely that the accumulation of this amine was influenced in the same way as its precursor a methyl-DA.

The last-mentioned observations should be viewed in conjunction with the results obtained in the blood pressure experiments. In animals pretreated with Ro 4-4602, the hypotensive effect of a MD was inhibited. On the other hand, a MD retained its blood pressure lowering activity after pretreatment with MK 485. It may be pointed out that the preventive activity of Ro 4-4602 was evident in spite of a considerably larger dose of a-MD used in these experiments than in those with MK 485. While indicating that the decarboxylation products of a MD play an important role in mediating the hypotensive action of the drug, these results do not support the view that its main action is located in the peripheral sympathetic nerves (DAY & RAND 1964 cf. reviews by CROUT 1966 MUSCHOLL 1966 STONE & PORTER 1966 & 1967 SOURKES & RODRIGUEZ 1967 KOPIN 1968). In this connection it may further be noted that there is no clear-cut time relation between the NA depletion in peripheral tissues and the decrease in blood pressure after a single dose of a-MD, the latter effect being shorter lasting than the former (HENNING 1967). This is also shown in the present study: no significant depletion of femoral muscle NA was observed at the time of maximal hypotensive effect. Further, there seems to be only a slight impairment of peripheral adrenergic mechanisms at this time (HENNING & SVENSSON 1968). Following the administration of a-methyl-DA or a methyl-NA, peripheral stores of NA are depleted at least as much as after a-MD. Yet, the two a-methylated amines lack antihypertensive properties in the rat (BRUNNER *et al.* 1966 & 1967 HENNING 1967). It would appear that the theory of false transmission, in its simplest outline, does not adequately explain the mode of action of a MD. A better understanding may be provided if this theory is extended to include central nervous neurons. Several indications of an impairment of central nervous mechanisms may be quoted (see review by SOURKES 1965) and recent studies have given direct evidence that the

hypotensive effect of a MD in the cat may be, at least in part, of central origin (HENNING & VAN ZWIETEN 1968).

The present results probably explain the discrepancy between the previously mentioned studies on the interaction of decarboxylase inhibitors with the antihypertensive effect of a MD. In the investigation by SJOERDSMA *et al* (1963) MK 485 was unable to prevent the fall in blood pressure after a MD in spite of a marked reduction in the urinary excretion of α -methyl-DA. However as shown by PORTER *et al*. (1962) and in the present study MK 485 is not an effective inhibitor of brain decarboxylase. On the other hand NSD 1039 was shown by DAVIS *et al* (1963) to inhibit significantly this enzyme in the brain and to block the hypotensive action of a MD.

It is of interest that the results of this study indicate that decarboxylase inhibition alone may result in a decrease in blood pressure. A significant effect was observed 12–48 hours after the beginning of the treatment with Ro 4-4602. This is in contrast to previous studies with MK 485 in various species (for references, see SJOERDSMA *et al* 1963), with NSD 1039 in rats (DAVIS *et al* 1963) or with NSD 1055 in humans (LEVINE & SJOERDSMA 1964). No reports seem to be available as to the effect of Ro 4-4602 in this respect. It is possible that the repeated large doses used in the present study with this potent inhibitor produced sufficient inhibition of monoamine synthesis to result in the functional impairment of the peripheral or central neurons, or both, and hence a decrease in blood pressure. The fact that MK 485 was less active than Ro 4-4602 in this respect supports the view of a central effect. Inhibition of tyrosine hydroxylase by means of α methyl *p*-tyrosine lowers the blood pressure in hypertensive rats with a time course similar to that observed after Ro 4-4602 in the present study (LAVERY & ROBERTSON 1968; HENNING unpublished results). Inhibition of the synthesis may also explain the prolonged hypotensive effect of decarboxylase inhibitors in combination with a MD. However since this effect was observed after both types of inhibitors, a delayed effect of a MD may have contributed to the response. Some of the drug may still be present in the blood stream or tissues at a time when the inhibition of decarboxylase is wearing off.

In conclusion, the following observations of the present study appear to be the most relevant. When the decarboxylation of a MD was prevented both centrally and peripherally by Ro 4-4602, the hypotensive action of a MD was blocked. When the decarboxylation of a MD was prevented peripherally but not centrally by MK 485 the hypotensive action of a MD persisted. It may be concluded from these observations that decarboxylation of α -MD in the central nervous system is a prerequisite for its hypotensive action. Further experiments are required to settle the

question, whether α -methyl-DA or α -methyl-NA, or both, are responsible for the central effect, or if their action is mediated by a false transmitter mechanism in the brain. Needless to say a peripheral action, although insufficient by itself, may play a contributory role in the hypotensive effect.

Summary

The influence of pretreatment with two inhibitors of DOPA decarboxylase on the hypotensive response to α -methyl-dopa (α MD) was studied in rats. In one series of experiments, *seryl* 2,3 4-trihydroxy benzylhydrazine (Ro 4-4602) was given in three doses of 200 mg/kg i.p. at intervals of 2 hours, the first dose being given 30 min. before α MD 200 mg/kg i.p. The hypotensive effect of α -MD was completely inhibited by this pretreatment, which also significantly reduced the accumulation of α -methyl-dopamine (α -methyl-DA) in the heart and the brain after α -MD. Further the depletion of brain dopamine (DA) and of heart noradrenaline (NA) by this drug was prevented by Ro 4-4602.

Another decarboxylase inhibitor α -hydrazino- α -methyl- β -(3 4-dihydroxyphenyl)propionic acid (MK 485) was given i.p. in four doses of 100 mg/kg at intervals of 2 hours, the first dose being given 30 min. before α MD 100 mg/kg i.p. This pretreatment had no effect on the hypotensive response to α -MD but inhibited the accumulation of α -methyl-DA and the depletion of NA in the heart. However MK 485 did not prevent the accumulation of α -methyl-DA or the depletion of DA and NA in the brain.

It is widely accepted that the antihypertensive property of α MD is mediated by its amine metabolites, α -methyl-noradrenaline or α -methyl-DA, or both, which act as "false transmitters" in the peripheral sympathetic nerves. In the present study inhibition of the decarboxylation of α -MD in the peripheral sympathetic system only did not influence the hypotensive action of the drug, whereas this effect was abolished when the inhibition was extended to the central nervous system. While not excluding the contribution of peripheral effects, these results point to the involvement of central nervous mechanisms in the action of α MD on blood pressure.

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Uptake of Mercury Vapour in Blood in Vivo and in Vitro from Hg-containing Air

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The uptake of mercury vapour from the respiratory tract has been investigated both in animals and man by several investigators. The uptake varied from 25 to 100 % of the inhaled mercury vapour (GERSTNER 1931 FRASER 1934 SHEPHERD *et al* 1941 GAGE 1961 HAYES & ROTHSTEIN 1962).

It has been questioned whether the uptake of mercury occurs from the mucous membranes of the respiratory tract or through the alveoli of the lungs (SPIEGEL 1957 HAYES & ROTHSTEIN 1962 TESSINGER & FISEROVA BERGEROVA 1965).

In experiments on four subjects who inhaled air with a mercury content of 100 µg per m³ for 7 hours, TESSINGER & FISEROVA BERGEROVA (1965) found a constant Hg retention of 76 % when the subjects inspired through the nose and expired through the mouth. The retention observed was about 20 % lower with oral respiration. The authors concluded that the mercury probably reacted with and was taken up exclusively by the respiratory mucosae. Histochemical experiments performed to confirm this assumption were unsuccessful (FISEROVA BERGEROVA 1966).

NIELSEN KUDSK (1965a & b) determined the size of a dead space for mercury vapour uptake from the respiratory tract and found that this was approximately the same as the physiological dead space. The dead space for mercury was found to be dependent on the size of the tidal volume, in the same way as the physiological dead space. Alveolar air was found to be virtually mercury-free. On the basis of these results it seemed reasonable to assume that the absorption of mercury vapour in normal subjects predominantly occurs from the alveoli of the lungs.

NIELSEN KUDSK (1965b 1966) reported that small amounts of ethyl alcohol given by mouth produced an inhibition of the pulmonary absorption of mercury. At a blood-alcohol concentration of about 0.02 % the

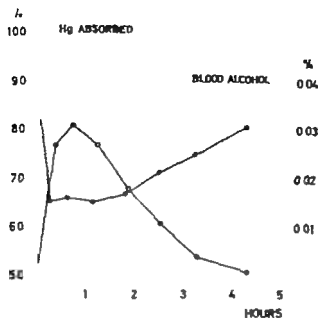


Fig. 1. Curves showing the pulmonary mercury absorption and blood-alcohol concentrations related to time in a male subject (61 kg) who at zero time, ingested 24 grammes of ethyl alcohol.

absorption fell by 25 %. When the blood-alcohol concentration was doubled, the fall increased to 30 %. A fourfold increase in the concentration did not cause any further inhibition of the mercury absorption. Fig. 1 shows the relationship between the blood-alcohol concentration and the pulmonary absorption of mercury vapour in a human volunteer (from NIELSEN KUDSK 1966).

The assumption that mercury vapour is absorbed from the lung alveoli is further supported by the demonstration of a relatively rapid uptake and oxidation of mercury vapour in blood *in vitro* (CLARKSON *et al.* 1961). Using the same equilibration technique as CLARKSON *et al.* in preliminary *in vitro* experiments, the present author found lower values for the rate of mercury-vapour uptake in whole blood and plasma. It was further demonstrated that increasing concentrations of ethyl alcohol caused a progressive inhibition of the uptake of mercury vapour in blood *in vitro* at physiological oxygen pressures (NIELSEN KUDSK 1966).

In order to confirm previous results on mercury-vapour absorption from the lungs in human subjects, a study was made using a more accurate technique. Experiments were also performed in an attempt to clarify the mechanism by which mercury vapour is taken up and oxidised by blood *in vitro* and also to explain the inhibitory effect of ethyl alcohol.

Material and Methods

The ratio of the mercury concentration in the expired air to that in the inspired air was determined in four subjects. Various constant concentrations in the inspired air in the range of 50–350 $\mu\text{g Hg per m}^3$ were used. The method used for direct and continuous graphical recording of the average concentration of mercury vapour in the expired air by means of ultraviolet photometry has previously been described (NIELSEN KUNSK 1965b). The minute volumes in the experimental periods of 2 minutes each were kept as constant as possible (approx. 7 litres). The number of expirations were recorded and the average tidal volumes calculated. The subjects inspired through the nose and expired through the mouth in most of the experiments. In several cases, respiratory valve system was introduced which allowed respiration through the mouth only.

The *in vitro* uptake of mercury vapour in blood and plasma was investigated by means of a simple equilibration technique similar to that used by CLARKSON *et al.* (1961). Human blood was obtained by venepuncture immediately before the experiments and stabilised with heparin. 3 ml samples of either whole blood or plasma were placed in 15 ml Warburg vessels, containing 0.1 ml recently double-distilled mercury in the centre well and side arm. The vessels were placed in a constant-temperature water bath at 37°C and equilibration took place in an atmospheric air phase at normal pressure. The vessels were shaken during the experimental periods, which varied from $\frac{1}{2}$ to 13½ hours.

Other experiments were carried out in order to study the mercury uptake in samples of whole blood, plasma, erythrocyte suspensions or haemolysates to which different alcohols, 3-amino-1,2,4-triazole, glucose and methylene blue had been added.

Blood samples of 4 ml were centrifuged at 3000 r.p.m. for 15 minutes, and the compounds to be tested were dissolved in a small volume of physiological saline and added to the plasma phase. Immediately afterwards the cell and plasma phases were carefully mixed, and 3 ml samples of the mixture transferred in the Warburg vessels and equilibrated with mercury vapour as described above. The equilibration times were either 3 or 6 hours. The cell volume in the blood used for the experiments was in the range of 45–50%.

Erythrocyte suspensions were prepared by removing the plasma and white cells from 4 ml blood samples by centrifugation, washing the erythrocytes 3 times with 6 ml physiological saline and finally substituting the plasma volume removed by saline. Some experiments were also performed with toluene-extracted erythrocyte haemolysates. The erythrocytes were washed as described above, haemolysed in an equal volume of water and the solution was extracted with toluene. After removal of the toluene extract, the original sodium chloride concentration was restored.

The air phase in the Warburg vessels was probably completely saturated with mercury vapour during the experiments. The use of only 0.1 ml mercury in the central well of the vessels did not lower the uptake of mercury in the blood samples or in an acid potassium permanganate solution. Calculated on the basis of the mercury-vapour pressure at 37°C the concentration at saturation in air is 51 mg Hg per m^3 .

The mercury uptake in blood and blood preparations during the equilibration period was determined by Hg analysis on 2 ml samples. The method of NIELSEN KUNSK (1964) was modified in that the digestion of the samples was performed with sulphuric acid and potassium permanganate only.

Digestion procedure. 2 ml blood is pipetted into a 500 ml Kjeldahl flask and the pipette is rinsed with 2 ml saline, which is added to the flask. After addition of 8 ml concentrated sulphuric acid, the flask is left standing for 15 minutes. 10 ml 6% potassium permanganate solution is then added while the flask is cooled under the tap. After standing at room temperature for 20 minutes 1 gramme of potassium permanganate is added to the flask

and a Liebig condenser inserted. The contents of the flask are then boiled for 45 minutes, and 10 ml 6% potassium permanganate followed by 10 ml water are added. The contents are vigorously boiled for another 45 minutes. After cooling to room temperature the digest solution is reduced and analysed as previously described.

Results

In vivo experiments

Table I shows the results of a series of determinations of the ratio (expressed as a percentage) between expired and inspired mercury vapour in four subjects receiving various Hg concentrations in the inspired air. This ratio is a measure of the fraction which the dead space for Hg absorption constitutes of the average tidal volume, which was determined

Table I

Results of experiments showing the ratio (given as a percentage) between expired and inspired mercury vapour related to the average tidal volume at various mercury concentrations in the inspired air in four subjects.

- I Mercury-vapour concentration in inspired air $\mu\text{g per m}^3$
 II Mercury-vapour concentration in expired air $\mu\text{g per m}^3$
 III Expired mercury as percentage of inspired mercury
 IV Average tidal volumes in litres (ATP).

I	II	III	IV	I	II	III	IV
A, male subject				C, female subject			
54.7	6.8	13.9	1.27	54.7	14.5	25.6	0.54
53.6	7.6	13.7	1.27	55.2	14.5	26.3	0.52
95.0	12.8	13.5	1.27	95.0	24.7	26.0	0.54
101	15.3	15.2	1.17	102	24.7	24.2	0.54
192	26.0	13.6	1.27	187	45.0	24.1	0.52
202	25.5	12.6	1.40	202	46.6	23.1	0.56
354	45.0	12.7	1.56	335	86.0	25.7	0.52
361	47.0	13.0	1.27	362	81.0	22.4	0.54
B, male subject				D, male subject			
54.7	11.1	20.2	0.88	49.3	13.6	27.6	0.54
54.7	11.5	21.0	0.83	51.2	13.0	25.4	0.54
98.0	14.5	14.8	1.00	102	27.1	26.6	0.58
99.0	14.5	14.7	1.00	102	27.2	26.7	0.56
211	36.0	17.1	0.93	118	62.0	28.4	0.56
215	43.0	20.0	0.88	218	60.0	7.5	0.56
366	57.0	15.6	1.08	366	100	27.3	0.58
345	50.0	14.5	1.08	369	98.0	26.6	0.58

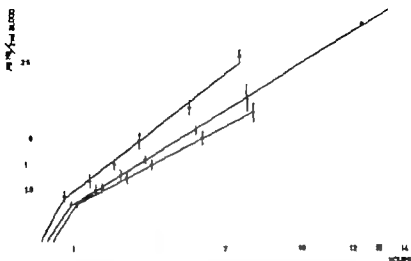


Fig. 2. Graphs showing the *in vitro* mercury-vapour uptake related to time in 2 ml blood samples from three subjects. For practical reasons, the curves are displaced 15 minutes to each other along the baseline. Each point, with the corresponding standard deviation, represents the average of six experimental results.

simultaneously during the experimental period. The subjects inspired through the nose and expired through the mouth. The ratios of expired to inspired Hg were determined with an accuracy of more than $\pm 10\%$. The errors involved in the average tidal volumes did not exceed $\pm 7\%$.

In several similar experiments the subjects respired through the mouth only by means of a valve system. The ratio between expired and inspired Hg was found to be a few per cent higher in these experiments, but when correction was made for the dead space of the valve system, the results were in agreement with those in table 1. This is at variance with the findings of TESSINGER & FISEROVA BERGEROVA (1965) viz. that the mercury retention was 20% lower with oral respiration than with nasal inspiration and oral expiration.

When the size of the dead space for Hg absorption is estimated from the figures given in table 1 (columns III and IV), it is obvious that this is of the same order of magnitude as would be expected for the physiological dead space according to ASMUSSEN & NIELSEN (1956).

In vitro experiments

The results of a study of the mercury-vapour uptake *in vitro* in whole-blood samples from three subjects as related to time are shown in fig. 2.

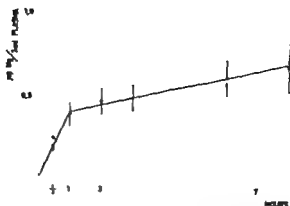


Fig. 3 The *in vitro* uptake of mercury vapour in 2 ml samples of human plasma related to time. The plasma samples were obtained from three subjects. Each average value, with its standard deviation, is based on five experiments.

The equilibration took place in an atmospheric air phase at 37° and normal pressure. The uptake appeared to proceed at two rates. During the first hour the rate was approximately 0.4 µg per hour per ml, while during the remaining part of the experimental period the rate of uptake was constant for each subject's blood, but varied in the blood from different individuals between 0.13 and 0.20 µg per hour per ml.

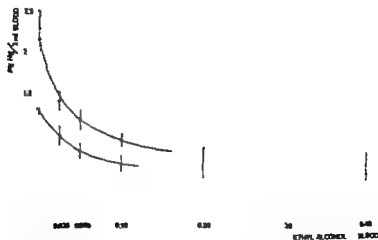


Fig. 4. Curves showing the progressive inhibitory effect of increasing ethyl alcohol concentrations on the *in vitro* uptake of mercury vapour in human blood. The equilibration times used were 3 (lower curve) and 6 hours (upper curve). The blood samples were obtained from four subjects. The individual points of the lower curve represent the average of 14 and those of the upper curve, the average of seven experimental results. The standard deviations are shown as vertical lines.

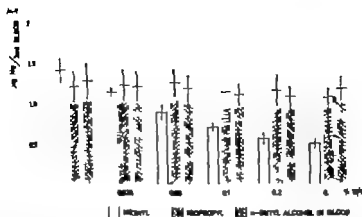


Fig. 5 Graphical representation of the influence of increasing concentrations of methyl, isopropyl and n-butyl alcohol on the *in vivo* uptake of mercury in blood. The number of experiments with these alcohols were 7, 7 and 5, respectively. The blood samples originated from four subjects. The calculated standard deviations are also shown. The equilibration times used were 3 hours.

The *in vitro* uptake of mercury vapour in human plasma related to time is shown in fig. 3. The uptake occurred at two rates. During the first hour the rate was $0.20 \mu\text{g}$ per hour per ml, followed by a slower rate of about $0.02 \mu\text{g}$ per hour per ml.

In similar experiments lasting up to 3 hours the uptake of Hg in erythrocyte suspensions from different subjects was $0.17\text{--}0.23 \mu\text{g}$ per hour per ml. This is a somewhat higher rate of uptake than was expected in view of the values found in whole blood and plasma. The addition of 0.25% glucose did not affect the rate of Hg uptake.

The mercury uptake in toluene-extracted erythrocyte haemolysates determined in a similar manner was approximately $1 \pm 0.4 \mu\text{g}$ Hg per hour per ml. Equilibration times of 2–5 hours were used.

Fig. 4 shows the progressive inhibitory effect of increasing ethyl alcohol concentrations on the uptake of mercury vapour in samples of human blood. In the equilibration experiments of 3 hours duration the inhibition reaches a maximum of approximately 60% at a blood alcohol concentration of about 0.2% . At a slightly higher alcohol concentration the maximal inhibition in the 6 hours experiments is 70% .

From the results shown in fig. 5 it can be concluded that methyl alcohol exerts a smaller inhibitory effect on the mercury uptake in blood, and that isopropyl and n-butyl alcohols are without any significant effect.

In equilibration experiments extending over 3 and 5 hours it was shown that ethyl alcohol in concentrations of 0.2 and 0.4% w/v respectively

Table 2

Results of five experiments showing the mercury vapour uptake in 2 ml approx. 45% suspensions of human erythrocytes in 0.9% sodium chloride with and without addition of glucose, methylene blue (MB), 3-amino-1,2,4-triazole (AT) or combinations of these compounds. The equilibration times were 3 hours. The blood samples were obtained from two subjects.

	Mercury-vapour uptake, in μg	
	Average	Standard deviation
Without any addition	1.21	± 0.13
Glucose, 0.25 %	1.27	± 0.12
MB, $10^{-4}/16$ M	2.06	± 0.16
Glucose, 0.25 % MB, $10^{-4}/16$ M	11.7	± 0.9
Glucose, 0.25 %, AT 0.12 M	1.25	± 0.12
Glucose, 0.25 % MB, $10^{-4}/16$ M AT 0.12 M	11.5	± 0.8

produced a maximum inhibition of 50% on the mercury-vapour uptake in plasma samples. In toluene-extracted erythrocyte haemolysates these ethyl alcohol concentrations caused an 80–90% inhibition in the Hg uptake in experiments of 3 or 5 hours duration.

The results of experiments performed in order to investigate whether hydrogen peroxide-catalase complex I might be responsible for oxidation of mercury in erythrocytes are shown in table 2. According to TERPILY *et al.* (1961), the concentration of aminotriazole used in conjunction with methylene blue and glucose should be sufficient to cause a marked inhibition of catalase or the primary hydrogen peroxide-catalase complex.

Addition of methylene blue alone to the erythrocyte suspensions obviously increases the rate of mercury vapour uptake considerably. In combination with glucose, however methylene blue causes a very marked acceleration in the rate of uptake. Aminotriazole does not seem to influence the uptake of Hg, whether or not this is stimulated by methylene blue. It thus seems unlikely that hydrogen peroxide-catalase complex I or catalase itself plays any role in the oxidation of mercury in the erythrocytes.

Discussion

By means of a more accurate technique it has been established that the respiratory dead space for mercury-vapour absorption corresponds to the size of the physiological dead space within a wide range of concentra-

tions. The variations in the size of the dead space for Hg in the single subject are very small and mainly due to variations in the tidal volume. Exclusion of the nasal air passages in experiments with oral respiration only do not influence the measured size of the dead space for Hg. Previous direct measurements have shown that the mercury vapour retention in the nasal air passages and the oral cavity is negligible (NIELSEN KUDSK 1965 a). These results very strongly indicate a complete alveolar absorption of mercury vapour.

The partition coefficient for metallic mercury between lipoids and air at 40 is approximately 25 l (HUGHES 1957) and mercury vapour probably passes through the alveolar and capillary walls by simple diffusion. HORWITZ (1957) showed that mercury vapour passes through plant and bacterial membranes unchanged. The rate of Hg uptake in blood *in vitro* is sufficiently high to explain a complete alveolar absorption *in vivo*. Using ^{203}Hg , CLARKSON (1965) found an uptake *in vitro* of approximately 0.07 μg Hg per hour per ml blood, at 37 and at a mercury vapour concentration of 400 μg per m^3 of atmospheric air. In the *in vitro* experiments, the results of which are given in fig. 2, the uptake of mercury occurred at a concentration of 51 mg per m^3 of air. The average uptake was about 0.2 μg per hour per ml under otherwise similar conditions. A comparison of these results seems to indicate that the last value might possibly be near to a maximum rate of Hg uptake in blood under the experimental conditions used *in vitro*.

The experimental findings presented in fig. 2 and fig. 3 are at variance with the results published by CLARKSON *et al* (1961) and CLARKSON (1965) who reported a constant rate of mercury vapour uptake in whole blood *in vitro* during the entire experimental period. These authors did not investigate any individual variation in the rate of Hg uptake in blood from different subjects. CLARKSON *et al* (1961) also reported a uniform and much higher rate (0.14 $\mu\text{g}/\text{hour}/\text{ml}$) of Hg uptake in plasma under identical experimental conditions.

The present authors' results indicate that the high rate of mercury vapour uptake in plasma during the first hour is the cause of the rapid initial phase of uptake in whole blood. During the first hour the uptake in plasma is 50% of the Hg uptake in whole blood, while during the following hours, the plasma-uptake only constitutes 10–15% of the uptake in blood. This seems to imply that 85–90% of the mercury taken up by whole blood after the first hour is taken up by and probably oxidised in the erythrocytes.

HORWITZ (1957) showed that plant cells and bacteria are capable of oxidising mercury and CLARKSON *et al* (1961) reported that metallic mercury vapour is oxidised in the blood mainly in the erythrocytes, and

that the oxidised mercury then equilibrates with plasma. The oxidised mercury was, presumably as mercuric ions, bound chiefly to the sulphhydryl groups of haemoglobin and plasma albumin.

The mechanism by which mercury is oxidised in the blood and particularly in the erythrocytes is as yet not clarified.

The fact that methyl and ethyl alcohol, but not isopropyl and n-butyl alcohol inhibit the mercury uptake in blood raises the question as to whether hydrogen peroxide and catalase might be involved in the oxidation of mercury in the erythrocytes. MARGOLASH & NOVOGRODSKY (1958) showed that when a hydrogen peroxide-generating system, such as methylene blue and glucose (TEPHLY *et al* 1961), was added to erythrocytes, sufficient amounts of hydrogen peroxide-catalase complex I were formed to cause a considerable oxidation of methyl and ethyl alcohol. The hydrogen peroxide-catalase complex I showed a high affinity for these alcohols, but not for isopropyl or n-butyl alcohol. It has also been shown that this complex and hence catalase can be irreversibly inhibited by 3-amino-1,2,4-triazole, and that this inhibition can be prevented by ethyl alcohol, while catalase itself can be reversibly inhibited by amino-triazol (MARGOLASH *et al* 1960, TEPHLY *et al* 1961, COHEN & HOCHSTEIN 1964).

The experimental results presented in table 2 seem to rule out catalase as being of any importance in the oxidation of mercury. However hydrogen peroxide, which is probably generated in erythrocytes to which glucose and methylene blue have been added might be responsible either directly or indirectly for the markedly accelerated uptake seen here.

A normal generation of hydrogen peroxide in erythrocytes has never been directly demonstrated. Erythrocytes contain several compounds (hydrogen donors) which by auto-oxidation or by a coupled reaction with the oxygen of oxyhaemoglobin, could produce hydrogen peroxide. An example of this is ascorbic acid which probably causes a constant generation of small amounts of hydrogen peroxide in a coupled reaction with oxyhaemoglobin (FOULKES & LEMBERG 1948, MILLS 1957 & 1960). Small amounts of hydrogen peroxide are presumably destroyed mainly by a reaction with glutathione catalysed by glutathione peroxidase (MILLS 1957, COHEN & HOCHSTEIN 1963), by which oxidised glutathione is formed. In the presence of glucose, this compound is again reduced by means of glutathione reductase which is primarily NADPH-dependent, but probably to some extent capable of functioning with NADH (MILLS 1960). WAGENKNECHT & EIFLER (1962) have observed the existence of a NADH-dependent reductase.

In haemolysates and plasma, glutathione is oxidised at a fairly high rate (BEUTLER *et al* 1963). The reasons for this is not known. Hydrogen

peroxide generated from auto-oxidisable substances could be the oxidant responsible. The high rate of mercury uptake found in haemolysates may possibly be related to the same phenomenon.

More experiments are needed to clarify the possible roles of hydrogen peroxide and the coupled glutathione and carbohydrate metabolism in the *in vitro* uptake of mercury vapour in blood. The inhibitory effect of ethyl alcohol also requires further investigation.

Summary

By using a more accurate technique than previously used by the author it was established experimentally that the respiratory dead space for mercury vapour in human subjects corresponds to the physiological dead space. This finding strongly indicates a complete alveolar absorption of mercury vapour in the lungs.

The rate of uptake of mercury vapour *in vitro* in blood was determined in samples from individual subjects. A minor but distinct individual variation was found. Mercury was taken up in blood at a higher rate during the first hour. A high rate of uptake in plasma during that period was also demonstrated.

Results showing a pronounced inhibitory effect of ethyl and methyl alcohol on the *in vitro* uptake of mercury in blood are presented. Isopropyl and n-butyl alcohol did not cause a similar inhibition.

A possible involvement of the primary hydrogen peroxide-catalase complex or catalase only in the oxidation and uptake of mercury in erythrocytes was investigated, using aminotriazole as an inhibitor either alone or in combination with methylene blue and glucose as a hydrogen peroxide-generating system. Aminotriazole was found to be without effect. Methylene blue alone and especially in combination with glucose, caused a pronounced accelerated uptake of mercury in the erythrocytes.

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Factors Influencing the *In Vitro* Uptake of Mercury Vapour in Blood

By

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In a previous paper (NIELSEN KUDSK 1969), it was stated that the *in vitro* mercury-vapour uptake in whole blood from individual subjects was subject to a minor but distinct individual variation. It was also demonstrated that the rate of mercury uptake during the first hour was essentially higher than during the following hours. A marked inhibitory effect by ethyl alcohol on the *in vitro* uptake of mercury in the blood, plasma and particularly by erythrocyte haemolysates was observed.

A possible involvement of catalase in the oxidation and uptake of mercury in erythrocytes was investigated using 3-amino-1,2,4-triazole as an inhibitor either alone or in combination with methylene blue and glucose as a probable hydrogen peroxide-generating system. Aminotriazole was without any effect. Methylene blue alone and especially in combination with glucose produced a marked acceleration in the rate of uptake of mercury vapour in the erythrocytes.

Attention was thus directed to hydrogen peroxide as a compound which either directly or indirectly might be of importance for the oxidation of mercury in the erythrocytes.

The stimulating effect of methylene blue on the uptake of mercury vapour in blood has been further investigated. Experiments have been performed with menadione, which is known to cause a detectable formation of hydrogen peroxide in erythrocytes (COHEN & HOCHSTEIN 1964). A number of other experiments have been carried out, using various metabolic stimulants and inhibitors in order to demonstrate a possible relationship between the mercury uptake and oxidation in blood *in vitro* and the coupled glutathione and carbohydrate metabolism.

Material and Methods

The *in vitro* uptake of mercury vapour in blood to which various compounds had been added was investigated by means of a simple equilibration technique previously used by the author (NIELSEN KUDSK 1969).

Human blood was obtained by venepuncture immediately before the experiments and stabilised with heparin. Blood samples of 4 ml were centrifuged at 3,000 r.p.m. for 15 minutes, and the compounds to be tested were dissolved in a small volume of physiological saline and then added to the plasma phase. The cell and plasma phases were then carefully mixed and 3 ml samples transferred to 15 ml Warburg vessels containing 0.1 ml double-distilled mercury in the centre well and side arm. The vessels were placed in a constant temperature water bath at 37° and equilibrated in an atmospheric air phase and at normal pressure. The vessels were shaken during the period of equilibration. The cell volume in the blood used for the experiments was in the range of 45–50%.

The uptake of mercury vapour in 2 mM solutions of glutathione in physiological saline with and without addition of other compounds was studied in a similar way.

The mercury uptake in blood and glutathione solutions during the equilibration periods was determined by mercury analyses on 2 ml samples according to the method of NIELSEN KUDSK (1964) with the modifications of the digestion given in a subsequent paper (NIELSEN KUDSK 1969).

Results

Fig. 1 shows the results of a study on the effect of methylene blue on the *in vitro* uptake of mercury vapour in whole blood. Increasing concentrations of methylene blue cause a very marked acceleration in the rate of mercury uptake, which reaches a maximum of about 12.5 µg mercury per 2 ml per 3 hours at a methylene blue concentration in the blood of

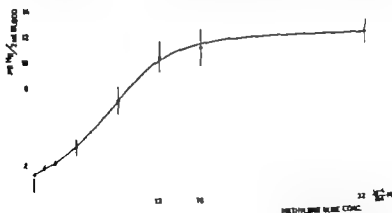


Fig. 1 Graph showing the accelerated rate of *in vitro* uptake of mercury vapour in blood caused by increasing concentrations of methylene blue. Each point represents the average value of six experiments. Standard deviations are shown as vertical lines. The blood samples originated from one subject, and the equilibration times were 3 hours. 0.4% glucose was added to all blood samples.

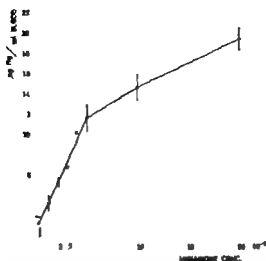


Fig. 2. Curve showing the influence of increasing menadione sodium bisulphite concentrations on the rate of the *in vivo* uptake of mercury vapour in whole blood. Each point shown with corresponding standard deviation, represents the average value of seven experiments. The blood samples were obtained from one subject, and the equilibration times were 3 hours. 0.25 % glucose was added to the samples.

$\frac{1}{2} \times 10^{-4}$ M. The curve has a definite resemblance to a sigmoid dose-response curve.

Increasing concentrations of menadione were found to produce an even higher degree of acceleration in the rate of mercury uptake in whole blood. Fig. 2 shows the results of a series of experiments with blood samples from one subject. At a menadione concentration of 2×10^{-4} M

Table 1

Results of six experiments showing the mercury-vapour uptake in 2 ml samples of whole blood to which were added menadione sodium bisulphite (MEN), ethyl alcohol (EOH) or both compounds in combination. Glucose was added to all samples. The equilibration times were 3 hours. The blood samples were obtained from one subject.

	Mercury-vapour uptake, in μ g	
	Average	Standard deviation
Glucose, 0.25 %	1.30	± 0.12
Glucose, 0.25 %, EOH, 0.4 % w/v	0.50	± 0.04
Glucose, 0.25 %, MEN 5×10^{-4} M	12.2	± 0.3
Glucose, 0.25 %, MEN, 5×10^{-4} M EOH, 0.4 % w/v	1.3	± 0.13

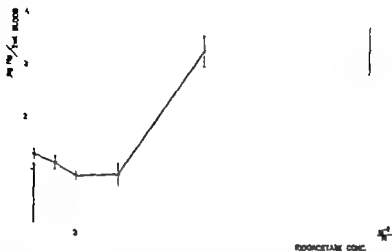


Fig. 3. Curve showing the dual influence of increasing iodo-acetate concentrations on the *in vitro* uptake of mercury vapour in human whole blood. Each \bar{x} is the average values, with corresponding standard deviation, is based on five experiments. The blood samples originated from three different subjects. The equilibration times with mercury were 3 hours.

the rate of mercury uptake in the blood is approximately 15 times the normal rate and has not yet reached a maximum. The shape of the curve is distinctly different from that shown in fig. 1. There is a linear relationship between the rate of uptake and concentrations of menadione up to 5×10^{-5} M.

The results given in table 1 show that the highly increased uptake in 2 ml blood samples per 3 hours caused by a concentration of 5×10^{-5} M menadione can be very effectively counteracted by the addition of 0.4% w/v ethyl alcohol.

Fig. 3 shows the results of five equilibration experiments with blood samples to which iodo-acetate had been added in increasing concentrations. Iodo-acetate obviously exerts a dual influence on the uptake of mercury vapour. Concentrations lower than $\frac{1}{2} \times 10^{-3}$ M inhibit the uptake slightly though distinctly while concentrations of $\frac{1}{2} \times 10^{-3}$ M and 10^{-3} M cause a mercury uptake in the blood of about 2.5 times the normal uptake.

The curve shown in fig. 4 represents the results of similar experiments with blood to which potassium cyanide was added in concentrations from $\frac{1}{2}$ to 4 mM. With increasing concentrations, progressive inhibition of the mercury vapour uptake in the samples takes place up to a maximum inhibition of approximately 60%. Concentrations of potassium cyanide 3 times as high as the highest given in the figure do not cause any further inhibition.

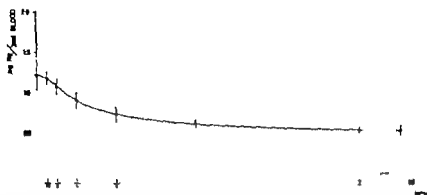


Fig. 4 Curve showing the decrease in the *in vitro* uptake of mercury vapour by blood caused by increasing potassium cyanide concentrations. Each of the points from which the curve is drawn represents the average of five experiments with blood samples from four subjects. The standard deviations are shown. The equilibration times were 3 hours.

In similar investigations, it was found that hydroxylamine in conjunction with glucose produced a highly accelerated rate of mercury uptake in the blood in concentrations of from $\frac{1}{2}$ to about 1 mM. At the latter concentration, the uptake was approximately 8 times the normal uptake. Higher concentrations did not cause any further increase in the rate of uptake. An obvious increasing formation of methaemoglobin with increasing hydroxylamine concentrations occurred in the blood samples. Without the addition of glucose to the blood the effect of hydroxylamine was much less marked.

Sodium fluoride in concentrations over 4.5 mM exerted a less distinct stimulating effect on the mercury uptake in the blood. At a fluoride concentration of 18 mM the uptake was about twice as high as the normal uptake. With increasing fluoride concentrations an increasing light red colour was seen.

Addition of glutathione to blood samples also caused an acceleration in the rate of mercury uptake. A linear relationship between increasing glutathione concentrations and the rate of uptake was observed. At a glutathione concentration of 2 mM the uptake occurred at a rate of approximately 6.5 times the normal rate.

Sodium nitrite in concentrations from $\frac{1}{2}$ to $1\frac{1}{2}$ mM produced a visible increasing methaemoglobin formation in blood samples, but did not influence the rate of uptake of mercury vapour.

Ascorbic acid showed a less pronounced effect on the mercury uptake in whole blood. A concentration of 5 mM produced approximately a

Table 2

Results of experiments showing the mercury-vapour uptake in 2 ml samples of whole blood which were equilibrated with mercury in atmospheres of pure oxygen, air or pure nitrogen for 3 or 6 hours at 37°C and at normal pressure. Average values based on five experimental results and the corresponding standard deviations are given in μg . The blood originated from one subject.

Equilibration time, in hours	Oxygen	Air	Nitrogen
3	1.50 ± 0.09	1.56 ± 0.08	1.04 ± 0.05
6	2.94 ± 0.13	2.04 ± 0.21	1.61 ± 0.11

twofold increase in the rate of uptake as compared with that in samples without any addition of ascorbic acid.

The results of the study of the mercury vapour uptake in blood exposed to pure oxygen and nitrogen atmospheres as compared with the uptake when equilibrated in an air phase are shown in table 2. Pure oxygen produces a very distinct, but moderate stimulation of the rate of uptake. Oxygen is obviously not indispensable for the uptake and oxidation of mercury as evidenced by the relatively small reduction in the rate of uptake in blood in a nitrogen atmosphere.

Simple glutathione solutions in physiological saline take up mercury vapour at a fairly slow rate as shown in table 3. Ageing of the solutions increases the uptake. Addition of hydrogen peroxide causes a pronounced

Table 3

The uptake of mercury vapour in 2 ml 2 mM glutathione solutions in physiological saline with and without addition of hydrogen peroxide, potassium cyanide, ethyl alcohol (EOH) or combinations of these compounds. The equilibration times were 2 hours. Each of the average values, with corresponding standard deviation, is based on five experiments.

	Mercury-vapour uptake, in μg	
	Average	Standard deviation
Without any addition	0.08	± 0.03
H_2O_2 , 1.25 mM	6.94	± 0.45
H_2O_2 , 1.25 mM KCN 4 mM	0.13	± 0.02
H_2O_2 , 1.25 mM EOH 0.8 % w/v	4.91	± 0.29

acceleration of the rate of uptake. Potassium cyanide very effectively inhibits the high rate of uptake caused by hydrogen peroxide, whereas a fairly high concentration of ethyl alcohol only moderately decreases the rate. Hydrogen peroxide-containing physiological saline takes up mercury at a very slow rate.

Neither methylene blue, nor menadione in the concentrations used in the experiments reported above caused any increase in the uptake of mercury vapour in glutathione solutions.

Discussion

The addition of methylene blue to erythrocytes or blood probably causes a generation of hydrogen peroxide in the erythrocytes, provided glucose and oxygen are available (TEPHLY *et al* 1961 GIBSON 1963). The exact mechanism by which such a generation takes place is not fully clarified, but methylene blue accepts electrons and hydrogen from NADPH, probably by way of a diaphorase and presumably the NADPH dependent methaemoglobin reductase. By this means leuco-methylene blue is formed which is then auto-oxidised under the formation of hydrogen peroxide. The NADPH in this reaction is supplied from the pentose shunt, the activity of which is highly stimulated by methylene blue (BRIN & YONEMOTO 1958).

The hydrogen peroxide formed is probably destroyed predominantly by a reaction with glutathione catalysed by glutathione peroxidase (MILLS 1957 COHEN & HOCHSTEIN 1963) by which oxidised glutathione is formed. In the presence of glucose, oxidised glutathione is again reduced to glutathione by means of glutathione reductase, which is mainly NADPH-dependent, but to some extent probably capable of using NADH (MILLS 1960). Evidence has been found for the existence of a NADH-dependent reductase (WAGENKNECHT & EIFLER 1962).

The sigmoid-shaped curve shown in fig. 1 might imply an individual variation in the ability of the erythrocytes to respond to methylene blue stimulation, possibly because of changes in the erythrocytes with age. It is known that the activity of hexokinase is reduced and that of glucose-6-phosphate dehydrogenase rather markedly diminished with increasing age of the erythrocytes (MORTENSEN 1965). Both these changes result in a decreasing pentose-shunt activity and consequently in a decreasing capacity to respond to methylene blue stimulation.

According to a personal communication by L. V. BECK in rat erythrocytes, a methylene blue concentration of 10^{-5} M produces a maximum stimulation of the pentose-shunt activity. As seen from fig. 1 this concen-

tration is of the same magnitude as that which causes a maximum rate of mercury vapour uptake in human blood.

The methylene blue stimulation of the rate of uptake in blood could possibly be explained by an increased rate of production of hydrogen peroxide coupled with an increased rate of oxidation of glutathione and metallic mercury.

It is well known that potassium cyanide reacts with oxidised glutathione. The finding that potassium cyanide inhibits the mercury vapour uptake in blood and in hydrogen peroxide-treated glutathione solutions very effectively seems to imply that oxidised glutathione (possibly as a free radical) participates in the oxidation of mercury.

The steady state concentration of oxidised glutathione normally found in erythrocytes ($45 \pm 10 \mu\text{g}$ per litre cells, GÜNTHERBERG & ROST 1966) is sufficient to account for a possible participation of oxidised glutathione in the normal *in vitro* uptake and oxidation of mercury vapour in erythrocytes.

It has been demonstrated that menadione causes hydrogen peroxide generation in erythrocytes (COHEN & HOCHSTEIN 1964). The mechanism by which this takes place is presumably similar to that of methylene blue (CARVER & RYAN 1960, JAFFÉ & NEUMANN 1964). However, menadione also exerts other effects on the erythrocytes. Phosphoglyceraldehyde dehydrogenase is inhibited by menadione (HOFFMAN-OSTENBOM 1963), and it is also possible that menadione accepts electrons from donors other than NADPH-dependent methaemoglobin reductase.

These circumstances may possibly be responsible for the difference between the curves showing the rates of mercury vapour uptake in blood, related to increasing concentrations of either methylene blue or menadione (figs 1 and 2).

According to WEBB (1966), an iodo-acetate concentration of about 0.4 mM causes a complete block of the glycolysis in the erythrocytes due to a selective and complete inhibition of phosphoglyceraldehyde dehydrogenase. This is followed by a decrease in the NADH/NAD ratio. As a consequence the glutathione reductase activity is probably diminished and the rate of reduction of oxidised glutathione somewhat decreased. This might possibly be related to the suddenly occurring increase in the rate of mercury uptake in blood containing iodo-acetate at a concentration of 0.5 mM. A direct inhibitory effect of iodo-acetate on the NADPH dependent glutathione reductase also seems possible (VENNESLAND & CONN 1954).

The moderate increase in mercury uptake in blood caused by sodium fluoride may similarly be related to an inhibition of glycolysis. The fluoride anion inhibits the glycolytic enzymes enolase and phospho-

glucosylase and eventually causes a decrease in glutathione reductase activity. The colour change to light red seen in the blood samples is probably related to the formation of fluoride complex with catalase and methaemoglobin.

The decreased rate of mercury uptake seen in blood containing iodoacetate in concentrations lower than 0.25 mM may possibly be due to a partial blockage of the SH groups available for mercuric ions on proteins and especially glutathione, which reacts rapidly with iodoacetate. It is also known that iodoacetate rapidly displaces mercuric ions from SH groups. Iodoacetate does not react with oxidised glutathione (WEBB 1966).

Hydroxylamine is known to cause an increased rate of oxidation of glutathione and methaemoglobin formation in erythrocytes (SCHEUCH *et al* 1963). It is probable, but has not been directly proved that hydroxylamine in a coupled reaction with the oxygen of oxyhaemoglobin induces hydrogen peroxide formation (COHEN & HOCHSTEIN 1964, ROSTORFER & CORMIER 1957).

The increase in the *in vitro* uptake of mercury caused by ascorbic acid is probably due to a generation of hydrogen peroxide in a coupled reaction of this compound with oxyhaemoglobin.

The fact that glutathione oxidises fairly rapidly in plasma (BEUTLER *et al* 1963) may possibly be related to its stimulating effect on the mercury vapour uptake in blood. It is not known if this oxidation of glutathione is accompanied by the generation of hydrogen peroxide. When added to blood it also stimulates the pentose-shunt in an unknown manner possibly by an action on the erythrocyte membrane (PRANKERD 1961).

Sodium nitrite is well known as an oxidant which causes methaemoglobin formation. Blood samples treated with this compound do not show any changes in mercury vapour uptake, and methaemoglobin presumably plays no role in the oxidation of metallic mercury.

As shown in table 2, pure oxygen stimulates the *in vitro* uptake of mercury in blood. This could possibly be caused by a higher rate of generation of hydrogen peroxide from auto-oxidisable substances in the erythrocytes, such as ascorbic acid and some of the diaphorases. The pentose-shunt activity is favoured by high oxygen tensions (WHITE *et al* 1959). The decreased rate of uptake of mercury in blood in a nitrogen atmosphere may be due to a decreased rate of formation of hydrogen peroxide and oxidised glutathione.

The investigations seem to indicate that hydrogen peroxide and oxidised glutathione – possibly as a free radical – are of major importance in the oxidation and uptake of mercury vapour in blood *in vitro*.

The very pronounced inhibition caused by ethyl alcohol on the increased

uptake of mercury in menadione-stimulated blood samples (table 1) cannot be explained at present. As shown in table 3 ethyl alcohol induces a moderate inhibition of the mercury uptake in hydrogen peroxide-treated glutathione solutions. This is also seen in freshly prepared glutathione solutions and is presumably due to an unspecific anti-oxidant effect in an auto-oxidizable reaction between glutathione and oxygen. It seems unlikely that the pronounced inhibition seen in conjunction with the mercury uptake in menadione-stimulated blood samples and in haemolysates can be explained in this way. A more specific inhibition exerted by ethyl alcohol on glutathione peroxidase or a possible complex of this with hydrogen peroxide would be a more likely explanation.

It is known that anaesthetics inhibit certain brain diaphorases which catalyse the anaerobic oxidation of NADPH and NADH by electron acceptors, such as methylene blue and menadione (QUASTEL 1963). An inhibition of the NADPH-dependent methaemoglobin reductase might possibly explain the inhibition exerted by ethyl alcohol on the mercury uptake in the menadione-stimulated blood samples. Experiments in which the effect of ethyl alcohol on the menadione-stimulated activity in the pentose-shunt could be investigated would presumably clarify this assumption.

Summary

The influence of a number of factors on the *in vitro* uptake of mercury vapour in blood has been investigated in order to clarify the mechanism by which mercury is oxidized in blood.

The rate of mercury uptake in blood in a pure oxygen atmosphere is moderately increased but somewhat decreased in a nitrogen atmosphere when compared with the rate of uptake in an atmospheric air phase.

Increasing concentrations of methylene blue from $1/16 \times 10^{-4}$ to $1/2 \times 10^{-4}$ M induce a very pronounced acceleration of the rate of mercury uptake in blood up to a maximum of about 10 times the normal uptake in an atmospheric air phase. Menadione shows a similar but even more pronounced effect in concentrations from 1 to 20×10^{-3} M. Hydrogen peroxide generation coupled with an increased rate of oxidation of glutathione catalysed by glutathione peroxidase, is a possible explanation for the acceleration of mercury uptake in blood caused by these two compounds.

The menadione-stimulated uptake could be markedly inhibited by low concentrations of ethyl alcohol.

Iodo-acetate in concentrations lower than $1/2 \times 10^{-3}$ M slightly but distinctly inhibits the mercury uptake in blood whereas concentrations of

$\frac{1}{2} \times 10^{-3}$ and 10^{-3} M produce an uptake of approximately 2.5 times that of the normal.

Concentrations of potassium cyanide from $\frac{1}{2} \times 10^{-3}$ to 4×10^{-3} M cause a progressive inhibition of the mercury uptake in blood up to a maximum of about 60% which is very similar to the effect produced by ethyl alcohol. The influence of sodium nitrite, hydroxylamine, ascorbic acid, sodium fluoride and glutathione on the mercury-vapour uptake in blood has also been investigated.

The investigations point to hydrogen peroxide and oxidised glutathione as agents of importance in the oxidation and uptake of mercury vapour in blood. The way in which ethyl alcohol inhibits the uptake is still unknown. Some possible mechanisms are discussed.

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The Effect of Thioridazine and Promazine on the Isolated Contracting Rat Heart

By

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Several investigators have called attention to the risk of cardiac complications during treatment with phenothiazine derivatives (KELLY FAY & LAVERTY 1963 DESAUTELS, FILTEAU & ST JEAN 1964 POULSEN 1965 ORNING 1966 LINGJÆRD 1967). The most frequent complications are changes in the electrocardiogram (ECG) characterized by elongation of the PQ- and QT-intervals, lowering of the T wave and disturbances in the QRS complex (TEITELBAUM 1963).

Left bundle branch block, ventricular tachycardia and fibrillation have also been reported. These complications are most frequently associated with thioridazine administration (ST JEAN & DESAUTELS 1966), but ECG changes ascribed to chlorpromazine have previously been observed in animals (MOYER *et al* 1954), and more recently also in humans (POULSEN 1965). It has been suggested that sudden death of patients treated with these drugs are due to their cardiac effects. Although there is little doubt that ECG changes can occur in patients receiving phenothiazines, the evidence for fatal cardiac complications arising from these drugs have been questioned (WENDKOS 1967).

In the present study we have examined the effects of two phenothiazine derivatives on the performance and the ECG pattern of isolated, contracting rat hearts. The dose-response curves obtained with thioridazine were compared to those obtained with promazine which is a less potent psychotropic agent. Changes in the ECG comparable to those observed

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als and rapidly transferred to ice-cold saline. Cannulation of the aorta and the left atrium was performed with the hearts submerged in ice-cold saline to prevent air from entering the heart cavities or the coronary arteries. The perfusion procedure has been described previously (Byx 1965), (fig. 1). The advantage of this method is that the heart maintains its physiological pumping activity *in vitro*. Systolic pressure, rate and aortic output through the cannulated aorta may therefore be used as performance criteria. This method also allows continuous recording of coronary flow. In the present experiments the hearts were perfused with a Krebs-Ringer bicarbonate buffer gassed with O_2/CO_2 (95/5%) and containing 1.8 mg/ml glucose and 10 mg/ml bovine serum albumin (Fraction V Sigma). The perfusate temperature was kept at 32°. ECG tracings were obtained by attaching recording electrodes to the aortic cannula and the cannula draining the chamber in which the heart was mounted. The apex of the heart was in continuous contact with the buffer at the bottom of this chamber.

The electrodes were connected to Sanborn Model 320 DC amplifier recorder. Pressure was recorded by Statham P 23 Gb transducer connected to the other channel of the recorder via a Sanborn 350 carrier pre-amplifier.

The hearts were kept beating for 25 min. *in vitro* before the addition of thioridazine or promazine. The drugs were added at the top of the oxygenator in doses giving final concentrations of $5 \cdot 10^{-4}$ M, 10^{-5} M, $2.5 \cdot 10^{-5}$ M and $5 \cdot 10^{-5}$ M, respectively.

Only hearts which pumped more than 8 ml/min. into the reservoir R_3 (aortic output, see fig. 1) before the addition of the drugs have been included in the results.

Thioridazine HCl and promazine HCl were gifts from Sandoz and Norfarm, respectively. The drugs were dissolved in distilled water and added to the perfusate at the oxygenator part of the perfusion circuit, in order to allow mixing of the drugs with the perfusate before reaching the heart.

Results

The excised hearts resumed spontaneous contractions during the first 1-2 min. in the perfusator and the performance increased rapidly during the following 5 min. After this time the cardiac output increased slowly during the subsequent 30-40 min. After 40 min., the hearts either showed a slow decrease in performance or maintained a steady performance throughout the following 1-2 hrs. Fig. 2 illustrates the effect of four different concentrations of thioridazine on aortic output. $2.5 \cdot 10^{-5}$ M thioridazine caused a significant but partly reversible decrease in output, whereas $5 \cdot 10^{-5}$ M caused a rapid and irreversible deterioration. The decrease in aortic output was partly due to a decrease in rate of contraction (fig. 3), and partly to a decrease in contractile force (fig. 4 - evaluated as the maximum systolic aortic pressure) both of which decreased in parallel to the decrease in output.

A small and transient increase in coronary flow occurred following the addition of 10^{-5} M thioridazine, whereas $5 \cdot 10^{-5}$ M caused an irreversible decline (fig. 5). Promazine, like thioridazine, caused a decrease in cardiac output, rate and contractile force, and the potency of the two drugs as

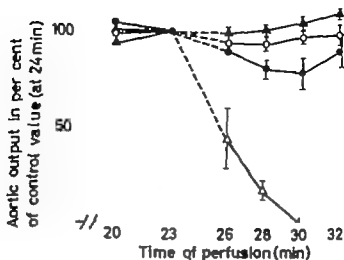


Fig. 2. Effect of four different concentrations of thioridazine on aortic output. Symbols: ▲—▲ $5 \cdot 10^{-4}$ M, ○—○ 10^{-3} M, ●—● $2.5 \cdot 10^{-3}$ M and △—△ $5 \cdot 10^{-3}$ M concentration. The drug was added at 25 min. Aortic output after addition of the drug is calculated in per cent of the output measured at 23 min. Each point represents 4–6 hearts (mean value \pm standard error of the mean).

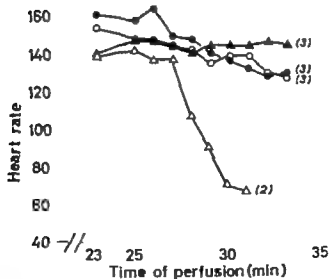


Fig. 3. Effect of four different concentrations of thioridazine on heart rate. (In brackets a number of experiments). Symbols: ▲—▲ $5 \cdot 10^{-4}$ M, ○—○ 10^{-3} M, ●—● $2.5 \cdot 10^{-3}$ M and △—△ $5 \cdot 10^{-3}$ M.

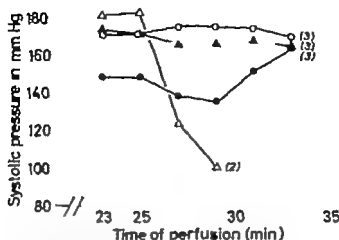


Fig. 4 Effect of four different concentrations of thioridazine on systolic pressure. (In brackets number of experiments). Diastolic pressure 58 mm Hg. Symbols Δ — Δ $5 \cdot 10^{-6}$ M, \bigcirc — \bigcirc 10^{-5} M, \bullet — \bullet $2.5 \cdot 10^{-5}$ M and \triangle — \triangle $5 \cdot 10^{-5}$ M.

cardiac depressants was similar when compared on a molar basis. Promazine however did not cause a reduction in coronary flow at these concentrations (fig. 6)

The ECG tracings obtained from the isolated rat hearts were characterized by a small P wave and a large R wave which usually fused with the T-wave (fig. 7). A depression of the R wave was the most striking change on addition of both thioridazine and promazine.

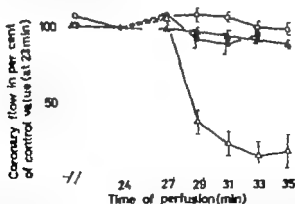


Fig. 5 Effect of four different concentrations of thioridazine on coronary flow. Conditions as in fig. 2. Coronary flow after addition of the drug (at 25 min.) is calculated as per cent of the flow measured at 4 min. Each point represents 4-6 hearts (mean value \pm standard error of the mean). Symbols Δ — Δ $5 \cdot 10^{-6}$ M, \bigcirc — \bigcirc 10^{-5} M, \bullet — \bullet $2.5 \cdot 10^{-5}$ M and \triangle — \triangle $5 \cdot 10^{-5}$ M.

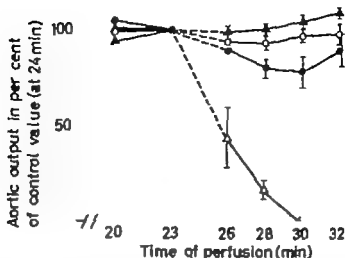


Fig. 2. Effect of four different concentrations of thioridazine on aortic output. Symbols Δ — Δ 5 $\times 10^{-5}$ M \circ — \circ 10 $^{-3}$ M \bullet — \bullet 2.5 $\times 10^{-3}$ M and \times — \times 5 $\times 10^{-4}$ M concentration. The drug was added at 25 min. Aortic output after addition of the drug is calculated in per cent of the output measured at 23 min. Each point represent 4–6 hearts (mean value \pm standard error of the mean).

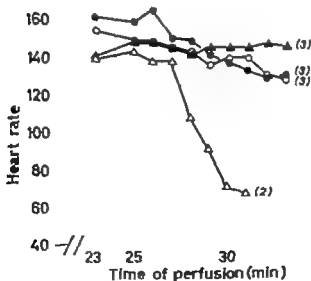


Fig. 3. Effect of four different concentrations of thioridazine heart rate. (In brackets number of experiments). Symbols Δ — Δ 5 $\times 10^{-5}$ M \circ — \circ 10 $^{-3}$ M \bullet — \bullet 2.5 $\times 10^{-3}$ M and \times — \times 5 $\times 10^{-4}$ M.

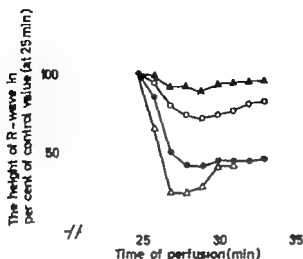


Fig. 8. Changes in the R-wave voltage of the electrocardiograms after addition of four different concentrations of thioridazine (1.25 mm). Height of R wave (voltage) is plotted as per cent of the height at the time of addition of the drug. Each curve represents mean values from 4-6 experiments. Symbols Δ — Δ $5 \cdot 10^{-6}$ M, \circ — \circ 10^{-5} M, \bullet — \bullet $2.5 \cdot 10^{-5}$ M and \triangle — \triangle $5 \cdot 10^{-5}$ M.

This depression was accompanied by an increase in the PQ-interval. At the highest concentrations of the drugs, the total ECG pattern including the T-waves was changed.

In some cases atrio-ventricular block and coupled ventricular extra

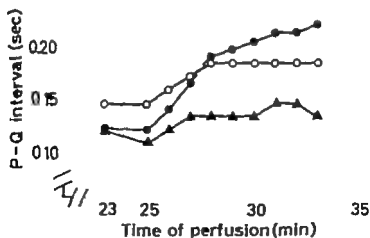


Fig. 9. Changes in the PQ-intervals of the electrocardiograms after addition of four different concentrations of thioridazine (1.25 mm). Each curve represents mean values from 4-6 experiments. Symbols Δ — Δ $5 \cdot 10^{-6}$ M, \circ — \circ 10^{-5} M, \bullet — \bullet $2.5 \cdot 10^{-5}$ M, \triangle — \triangle $5 \cdot 10^{-5}$ M.

systoles were observed ECG recordings from four typical experiments are shown in fig. 7. The effect of various concentrations of thioridazine on the R wave is shown in fig. 8 and the effect on the PQ-interval in fig. 9. Similar changes were found on addition of equimolar concentrations of promazine.

Discussion

The drug concentration used in the present experiments were calculated from a mol. wt. of 407 for thioridazine HCl and 320 for promazine HCl. The concentrations used thus equal about 2 µg, 4 µg, 10 µg and 20 µg per ml for thioridazine and 1.6 µg, 3.2 µg, 8 µg and 16 µg per ml for promazine. MELLINGER (1965) has shown that 100 mg per day of thioridazine given orally to patients results in a serum concentration of 0.7 µg per ml, and 200 mg per day results in serum levels of 1.3 µg per ml. In psychiatric treatment doses up to 600 mg per day are recommended and even higher doses have been used (KELLY, FAY & LAVERTY 1963). Sudden death have been described in patients given 900 mg thioridazine per day (ST. JEAN & DESAUTELS 1966). Serum levels comparable to at least the two lower concentrations used in the present experiment might therefore be expected in psychiatric patients treated with these drugs. The possibility of a species difference in drug tolerance, however, limits the usefulness of a direct comparison of the concentrations used in these experiments to serum concentrations of patients.

In our experiments ECG changes comparable to those which have been reported in patients treated with phenothiazines were observed at perfusate concentrations of about 10^{-5} M for both drugs tested. At this concentration no deterioration of cardiac performance was noted. Higher concentrations reduced aortic output and at $5 \cdot 10^{-3}$ M the hearts were unable to pump against the imposed hydrostatic pressure of 75 cm water. At this concentration thioridazine caused a rapid and irreversible decline in coronary flow. This reduction in coronary flow was not observed when the cardiac performance was reduced to a similar degree with promazine, and it is therefore likely that thioridazine at these concentrations constrict the coronary vessels in a more direct way. This property might possibly be of importance for the cardiotoxic effect reported for thioridazine when very high doses are given to psychiatric patients.

Several previous workers have studied the cardiovascular effects of phenothiazine derivatives in experimental animals *in vivo*. MELVILLE (1958) found that chlorpromazine 1–10 mg per kg caused a transitory fall in blood pressure, transient cardiac acceleration followed by bradycardia, and slight changes in the ECG (depression of T waves). MADAN (1963)

showed that thioridazine (5 mg and 10 mg per kg) caused hypotension, bradycardia and prolongation of the cardiac refractory period and conduction time in vagotomized, anaesthetized dogs. Prolongation of the PQ-interval and broadening of the QRS complex have been observed following 10 mg per kg of various phenothiazine derivatives given intravenously to dogs (SHARMA & ARORA 1961)

The present results show that both thioridazine and promazine have characteristic effects on the rat heart in the absence of nervous control. The rapid development of ECG changes after addition of the drugs suggests a direct action on the cardiac muscle membranes. In these experiments the ECG changes preceded the decline in cardiac performance following increasing concentrations of the drugs. If this is also the case *in vivo* regular ECG control might be useful in the prevention of fatal cardiac complications in patients receiving high doses of thioridazine.

Summary

The action of the two phenothiazine derivatives, thioridazine and promazine has been studied in an isolated contracting rat heart preparation. A concentration of 10^{-5} M of either drug caused slight ECG changes. At $2.5 \cdot 10^{-5}$ M both drugs depressed cardiac performance, and a concentration of $5 \cdot 10^{-5}$ M caused a rapid and irreversible deterioration. At the higher concentrations the total ECG pattern was changed and disturbances in heart rhythm occurred. While promazine ($5 \cdot 10^{-5}$ M) had no significant effect on the coronary flow thioridazine reduced the flow to a marked degree.

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Changes in Coronary Flow and ECG in the Isolated Perfused Rat Heart Induced by Phenothiazine Drugs

By

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Several investigators have described changes in the electrocardiographic pattern in patients treated with phenothiazine derivatives. The most common changes are prolongation of the duration of the QT-QRS-PR and "low voltage" but left bundle branch block, ventricular tachycardia and fibrillation are also seen (TEITELBAUM 1963 KELLY FAY & LAVERTY 1963 BAN & ST JEAN 1965 POULSEN 1965 LINGJÆRDE 1967). Sudden death of patients treated with phenothiazine derivatives have been related to these cardiac effects. Among the various phenothiazine drugs thioridazine has more often than the others been held responsible for severe cardiac complications. (REINERT & HERMANN 1960 RICHARDSON GRAUPNER & RICHARDSON 1960 DELAY & DENIKER 1961 WILSON & REEF 1964 GREINER & NICOLSON 1964 HOLLESTER & KOREK 1965 LINGJÆRDE 1967).

In a recent work LANDMARK, GLOMSTEIN & ØYE (1969) observed that thioridazine caused a rapid decrease in coronary flow in isolated perfused rat hearts. This effect was not shared by promazine, in spite of the fact that the latter agent was equipotent as a general cardiac depressant.

This reduction in coronary flow observed after thioridazine is in contrast to previous reports on the effect of other phenothiazine drugs on isolated rabbit and guinea pig hearts, where a transient increase in flow has been found (FEKETTE & BORSY 1962 COURFOISTE *et al.* 1953 MELVILLE 1954 SINGH, SHARMA & RATHORE 1967).

In the present investigation, the electrocardiogram and coronary flow of isolated perfused rat hearts were examined before and after addition of different phenothiazine derivatives known to have antipsychotic effects, and one without such effect, namely the antihistamine promethazine

Methods

Rats of both sexes, weighing about 250 g and of a local strain (originally Wistar) were used. The heart was excised under ether anaesthesia and immediately put into saline of 0. The aorta was cannulated (stainless steel cannula) and the heart perfused for 3 minutes at 20 with a modified Krebs-Ringer bicarbonate solution aerated with 95% O₂ and 5% CO₂. Following this wash-out period, the heart was transferred to a perfusion apparatus similar to that previously described by Øys (1965). The pressure of the aortic valves was maintained at 75 cm of water.

A "Mingograph 14" recorder was used for the ECG-registrations. One electrode is placed directly on the apex, and the other connected to the cannula situated in the aorta. Coronary flow was measured by collecting 5 ml of the fluid draining from the heart and recording the time with a stopwatch. Temperature was recorded with a milligraded thermometer placed in direct contact with the apex and continuously flushed by the perfusate leaving the heart. Care was taken to maintain constant temperature at this point, in order to avoid temperature dependent changes in the conduction velocity and coronary flow (unpublished results). The perfusion temperature was kept at 24. At this temperature the oxygen supply is adequate even in the absence of erythrocytes (Haugaas & Øys 1966). The perfusion medium contained the following ions (meq./l): Sodium 150, potassium 5.9, calcium 5.1, magnesium 2, chloride 127, phosphate H₂PO₄ 2.4, bicarbonate 24.8, sulphate 2.4. The medium also contained glucose 180 mg/100 ml. The total volume of the perfusate was always 40 ml, and it was continuously aerated with 95% O₂ and 5% CO₂. All the additions to the perfusate were made at the top of the oxygenator. The hearts were perfused for 16 minutes without any additions. Then the drug or control solution was added, and the perfusion usually carried on for another 15 minutes, the total perfusion time being 31 minutes. Coronary flow was measured and ECG-tracings made at regular intervals during the perfusion period. When the perfusion was finished, the hearts were blotted on filter paper and weighed. Coronary flow was calculated per gram wet weight (g.w.w.) in ml/g.w.w./min. 76 hearts are included in this series, 12 of which serve as controls. 12 hearts received chlorpromazine, 14 thioridazine, 13 levomepromazine, 11 perphenazine, 8 prochlorperazine and 7 promethazine. Each heart represents one concentration of the drug. The controls received either distilled water or solution of the corrigentia of the respective commercial drug preparation. The drugs used were the following commercial preparations: Largactil® (chlorpromazine), Nozinan® (levomepromazine), Trilafon® (perphenazine), Sicutil® (prochlorperazine) and Phenergan® (promethazine). Melleril® (thioridazine) was made available from Sandoz through the department of Pharmacology University of Oslo, and dissolved in distilled water.

Results

The isolated hearts resumed spontaneous contractions during the first 30 sec. in the perfusion circuit. During the first 2-3 minutes the contractions were irregular but after 3 min. all the hearts included in the present series had established a regular sinus rhythm.

A. Effects of phenothiazine drugs on coronary flow

After 5 minutes in the perfusion apparatus the hearts had a coronary flow between 6.8 and 8.1 ml/g.w.w./min. In all the control hearts the

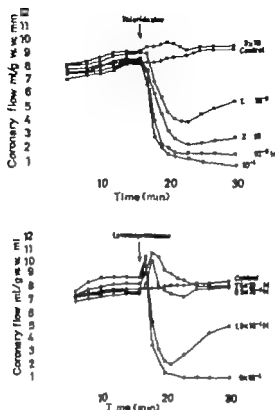


Fig. 1 & b.

a. Time curves showing changes in the coronary flow in the isolated rat heart following addition of thioridazine. The drug was added in different quantities giving different molar concentrations in the perfusate after 16 min. of perfusion. Each curve represents one heart and one concentration

b. Time curves showing changes in the coronary flow following addition of levomepromazine. Conditions as in fig. 1a.

coronary flow increased slightly during the 31 minutes of perfusion. The effect of phenothiazine drugs on coronary flow is shown in figs. 1 and 2. In low concentrations, these drugs caused a slight and transient increase in coronary flow (fig. 1). In high concentrations, however, all the drugs decreased the coronary flow but for most drugs this decrease was preceded by a transient increase (fig. 1b). When compared on a molar basis thioridazine was almost ten times more potent in reducing coronary flow than levomepromazine (table 1). The relative order of potency in this respect was 1) thioridazine 2) perphenazine 3) prochlorperazine 4) chlorpromazine 5) levomepromazine 6) promethazine. In so far as the

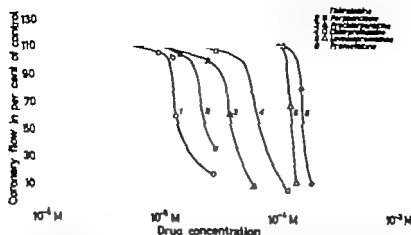


Fig. 2. Dose-response curves showing the effect of thioridazine, perphenazine, prochlorperazine, chlorpromazine, levomepromazine and promethazine on the coronary flow in the isolated rat heart. Coronary flow was recorded 14 min. after addition of the drug, and calculated as per cent of the flow at the time of addition of the drug.

transient increase in flow was concerned, the drugs also differed in potency. Levomepromazine was unique in causing a transient increase in all concentrations tested (ranging from $4.5 \cdot 10^{-6}$ M to $1.5 \cdot 10^{-4}$ M).

B Effects of phenothiazine drugs on the electrocardiogram

The control hearts maintained normal sinus rhythm without ECG alterations throughout the perfusion period. All the phenothiazine drugs

Table 1

The table shows the concentrations of different phenothiazine drugs which will reduce the coronary flow (c. fl.) in the isolated rat heart to a minimum of 90%, 50% and 25% of the flow before addition of the drug.

Phenothiazine drug	Concentrations which reduce c. fl. to 90%	Concentrations which reduce c. fl. to 50%	Concentrations which reduce c. fl. to 25%
Thioridazine	$1.35 \cdot 10^{-5}$ M	$1.4 \cdot 10^{-5}$ M	$1.9 \cdot 10^{-5}$ M
Perphenazine	$2.0 \cdot 10^{-5}$ M	$2.3 \cdot 10^{-5}$ M	$2.9 \cdot 10^{-5}$ M
Prochlorperazine	$3.6 \cdot 10^{-5}$ M	$3.9 \cdot 10^{-5}$ M	$4.7 \cdot 10^{-5}$ M
Chlorpromazine	$4.6 \cdot 10^{-5}$ M	$5.1 \cdot 10^{-5}$ M	$6.9 \cdot 10^{-5}$ M
Levomepromazine	10^{-4} M	$1.2 \cdot 10^{-4}$ M	$1.3 \cdot 10^{-4}$ M
Promethazine	$9.0 \cdot 10^{-5}$ M	$1.2 \cdot 10^{-4}$ M	$1.7 \cdot 10^{-4}$ M

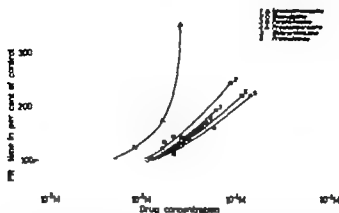


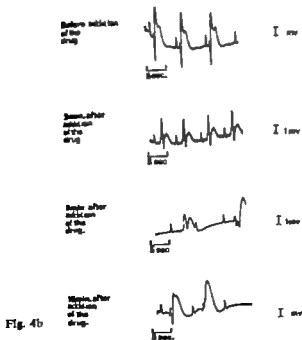
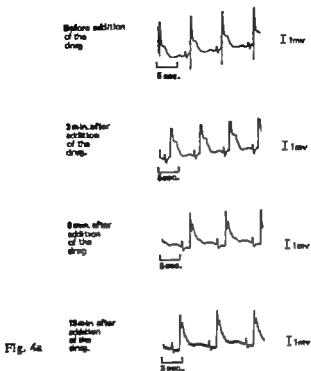
Fig. 3 Dose-response curves showing the effect of levomepromazine, thioridazine, perphenazine, prochlorperazine, chlorpromazine and promethazine on PR-interval in ECG recordings 8 min. after addition of the drug. PR-interval at this time is calculated as per cent of the PR-interval at the time of addition of the drug.

tested caused a dose-dependent increase in the PR interval, as shown in fig. 3. Further increase resulted in partial AV-block and still higher concentrations in total AV block. Levomepromazine induced all these changes in lower concentrations than the other drugs. All the drugs tested caused prolongation in durations of QT and QRS, reduction in the height of the R-wave, as well as reductions of the P and R frequencies (table 2).

Table 2

The table shows the concentrations of different phenothiazine drugs, which reduce the height of the R-wave to 85% of the height before addition of the drug, reduce P frequency to 85% of the frequency before addition and prolong the duration of QRS and QT to 120% of the durations before addition of the drug. No changes were observed in the controls. Only hearts with sinus rhythm are included.

Phenothiazine drug	Height of R-wave 85% of the height before add.	P-frequency 85% of the frequency before add.	QT-duration 120% of the duration before add.	QRS-duration 120% of the duration before add.
Thioridazine	$1.4 \cdot 10^{-6}$ M	$2.5 \cdot 10^{-3}$ M	$3.5 \cdot 10^{-3}$ M	$3.5 \cdot 10^{-3}$ M
Perphenazine	$1.7 \cdot 10^{-5}$ M	$3.5 \cdot 10^{-3}$ M	$3.5 \cdot 10^{-3}$ M	$2.5 \cdot 10^{-3}$ M
Prochlorperazine	$1.7 \cdot 10^{-5}$ M	$3.5 \cdot 10^{-3}$ M	$3.5 \cdot 10^{-3}$ M	$2.5 \cdot 10^{-3}$ M
Chlorpromazine	$1.4 \cdot 10^{-5}$ M	$3.5 \cdot 10^{-3}$ M	$3.5 \cdot 10^{-3}$ M	$3.5 \cdot 10^{-3}$ M
Levomepromazine	$1.7 \cdot 10^{-6}$ M	$2.5 \cdot 10^{-3}$ M	$3.0 \cdot 10^{-3}$ M	$2.5 \cdot 10^{-3}$ M
Promethazine	$5.0 \cdot 10^{-5}$ M	$5.9 \cdot 10^{-3}$ M	$3.9 \cdot 10^{-3}$ M	$5.9 \cdot 10^{-3}$ M



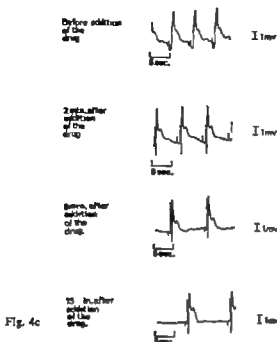


Fig. 4a, b & c. Examples of ECG-changes after addition of phenothiazine-derivatives to the isolated rat heart are given. In fig. 4a prolongation of PR-interval after addition of levomepromazine to a concentration of $1.9 \cdot 10^{-5}$ M in the perfusate is shown from top to bottom ECG before addition of levomepromazine, 2 min., 8 min. and 15 min. after addition of the drug. In fig. 4b ECG before and 2 min., 8 min. and 15 min. after addition of levomepromazine to the perfusate to a molar concentration $9.8 \cdot 10^{-5}$ M. Note the prolonged PR-interval 2 min. after addition, and the total AV-block with idioventricular rhythm (different ectopic foci) 8 and 15 min. after addition of the drug. (This concentration of levomepromazine does not reduce coronary flow). In fig. 4c the change in arterial pacer-maker 8 min. after addition of thioridazine to the perfusate to a molar concentration of $1.4 \cdot 10^{-5}$ M is shown. This concentration of thioridazine reduces coronary flow to 50% of the flow at the time of addition of the drug.

Promethazine was less potent than the other drugs in this respect. As long as sinus rhythm persisted, the R frequency was similar to the P-frequency but at higher concentrations partial or total AV block sometimes occurred with a further fall in R frequency.

Ectopic atrial beats were commonly observed with all the drugs in concentrations above 10^{-5} M. Upper nodal and lower-nodal rhythms (MARRIOT 1962) were occasionally observed at concentrations above $2 \cdot 10^{-5}$ M and ectopic ventricular beats occurred at concentrations about $4 \cdot 10^{-5}$ M and higher. Levomepromazine was somewhat more potent, and promethazine less potent than the other drugs in this respect. Most of

the drugs caused ectopic ventricular beats only at concentrations which also reduced the coronary flow. Levomepromazine, however, caused ectopic ventricular beats at concentrations lower than those necessary to reduce coronary flow. Some examples of the ECG changes are shown in fig. 4.

Discussion

The ECG-changes caused by phenothiazine drugs in the isolated rat heart correspond to those described in patients treated with phenothiazines, namely PR-QRS- and QT-prolongation, "low voltage" AV block, and ectopic ventricular and atrial beats. These changes as well as the changes in P frequency must be due to direct actions on cardiac conduction velocity, excitability and contractility ("low voltage") and are similar to those produced by quinidine (DESAUTELS, FILTEAU & ST JEAN 1964; BAN & ST JEAN 1965).

The present experiments show that high concentrations of phenothiazine drugs reduce the coronary flow in the heart deprived of nervous and hormonal control. In lower concentrations these drugs cause a transient increase in coronary flow, and the reduction in coronary flow at higher concentration is usually preceded by a transient increase. The fall in coronary flow is dose dependent and occurs at different concentrations for each of the drugs tested. There is no correlation between the reduction in coronary flow and the alteration in the ECG pattern, and no correlation between the effect of the drugs on the coronary vascular bed and their antipsychotic effect. (The doses commonly recommended in psychiatric treatment are approximately the same for chlorpromazine, thioridazine and levomepromazine. Lower doses are recommended for prochlorperazine, and much lower doses for perphenazine in order to obtain the same antipsychotic effect (LINGJÆRDE 1966).)

The reduction in coronary flow observed in the present experiments might be due to a direct action of the drugs on the coronary resistance vessels. However, reduction in coronary flow could also be secondary to changes in the contractile activity of the heart.

The work of LANDMARK, GLOMSTEIN & ØYE (1968) indicates that the decline in coronary flow caused by thioridazine is not secondary to the decrease in cardiac contractile activity. It is therefore likely that the decline in flow reflects a direct action on the tone of the coronary resistance vessels. In accordance with this interpretation is the fact that the effect of these drugs on coronary flow is also present in hearts maintained in an asystolic state by lowering the temperature to below 12° (unpublished observations).

One has to be cautious to make general conclusions from these experiments on the rat heart, but it is interesting to note that thioridazine, to which most fatalities have been ascribed is the most potent reducer of coronary flow. The drug concentrations used in the present experiments might be higher than the serum concentrations found in patients treated with thioridazine (MELLINGER 1965). The main conclusion, however is that a reduction in coronary flow occurs at thioridazine concentrations similar to or lower than those necessary to produce ECG changes. Such ECG-changes are common in patients treated with thioridazine, 400 mg/day or more. Levomepromazine, which is a less effective coronary vasoconstrictor has not been associated with sudden cardiac death to the author's knowledge, in spite of the fact that this drug was found to be more potent than thioridazine in causing ECG-changes. The possibility that certain phenothiazines might reduce coronary flow in patients treated with these drugs warrants further investigations *in vivo*.

Summary

Coronary flow and ECG have been recorded in the isolated perfused rat heart, before and after addition of one of the following phenothiazine derivatives: thioridazine, chlorpromazine, perphenazine, prochlorperazine, levomepromazine, and promethazine. The drugs caused a transient increase in coronary flow followed by a dose dependent decrease. When compared on a molar basis the following order of potency was found: 1) thioridazine 2) perphenazine 3) prochlorperazine 4) chlorpromazine 5) levomepromazine 6) promethazine. The drugs caused ECG-changes characterized by prolongation of PR-QT-QRS-durations, "low voltage" reduction in rate with sinus rhythm, further reduction with AV block and ectopic atrial and ventricular beats. There was no strict correlation between the reduction in coronary flow and the alterations in ECG-pattern, and it is suggested that the reduction in coronary flow is mainly due to a direct action of these drugs on the coronary resistance vessels.

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Excretion of Phenobarbitone in Urine after Intake of Large Doses

By

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(Received September 24, 1968)

BUTLER (1954) demonstration of p-hydroxyphenobarbitone (p-hydroxyphenemal) as an excretion product of phenobarbitone (phenemalum NFN) in dogs contributed to our knowledge concerning the mode of elimination of phenobarbitone. A similar conversion takes place in man (CURRY 1955a & b ALGERI & MCBAY 1956). However while dogs mainly excrete p-hydroxyphenobarbitone conjugated with glucuronic acid BUTLER (1956) found that humans excrete only part of the compound in a conjugated form, probably as a sulphate.

As early as 1918 LUCH & FEIGL showed that phenobarbitone is excreted at a very slow rate. SUDENDEY (1958) by paper chromatographic analysis, found that the excretion of p-hydroxyphenobarbitone could be followed almost as far as that of phenobarbitone, but he performed no adequate quantitative determination.

As no such investigation seems to have been undertaken on the long-term excretion in humans, we decided, when an opportunity offered to study the excretion of phenobarbitone and p-hydroxyphenobarbitone in a patient given large doses of phenobarbitone under controlled conditions.

Materials and Methods

Case report

The patient, a man aged 63 with myasthenia gravis (detailed case history: SECHER 1967) was subjected to prolonged curarising treatment, according to CHURCHILL DAVIDSON & RICHARDSON (1957). During this treatment he was kept asleep with phenobarbitone. Initially the patient was given narcodorm ® (salbomestum NFN), total 1 480 mg.

This was followed by the administration of phenobarbitone, though with supplementary doses of nembutal ® (mebomalinatrium NFN) 600 mg, until the full effect of phenobarbitone had been attained. Phenobarbitone was given by stomach tube in doses of 200 mg \times 4 for the first six days, 200 mg \times 3 during the next 48 hours, and, finally 200 mg \times 1 and 100 mg \times 2 during the last 24 hours. The doses given per day were administered at equal time intervals.

In addition, the patient received 620 mg tubocurarine (NFN) and 1500 mg pbenorgan ® (promethazinum chloridum NFN). The patient had not been given barbituric acid derivatives before.

While sleeping the patient received food and fluid partly by stomach tube and partly intravenously. A 24-hour urine volume exceeding 1500 ml was aimed at.

The patient woke up 64 hours after the last phenobarbitone injection without the treatment having had any effect.

Analytical Measurements.

Blood procedure. Samples were withdrawn daily at the same time for 31 days, the first time about 24 hours after the start of the anaesthesia.

The plasma is extracted with chloroform. The chloroform phase is evaporated and the residue extracted with boiling water. This is followed by extraction at pH 5.5 with chloroform and the organic phase is used for quantitative spectrophotometric measurement (DRANCO 1954). All the spectra are recorded within the range 200–350 nm by a self-recording spectrophotometer at pH 10, pH 2 and pH 13.5.

The amount of pentobarbitone in the sodium hydroxide phase is calculated and it is now possible to calculate the concentration of pentobarbitone in the borate phase (DRANCO 1954). Following correction for this, the amount of phenobarbitone in the borate phase is calculated.

Urine procedure. Phenobarbitone 24-hour samples were collected for 47 days, beginning on the first day of anaesthesia.

An aliquot of the samples is extracted three times with 1.5 volume of chloroform at pH 1.3. The further procedure is as described for blood.

A direct quantitative spectrophotometric analysis is impossible in most cases. A paper chromatographic purification is therefore performed (ALGERI & WALKER 1952). After the development of the chromatogram, phenobarbitone is eluted and the concentration measured spectrophotometrically.

In the presence of small quantities (less than 0.1 µg/ml urine) it is impossible to perform quantitative spectrophotometry. Therefore the eluate is used for two-dimensional thin-layer chromatography (silica gel-G, 0.25 mm). The chromatogram is developed by using the solvents described by WALKERSTEIN *et al.* (1958) and ALGERI & WALKER (1952), and sprayed with mercury sulphate-diphenylcarbazone reagent (SARTIN 1960). The amount of phenobarbitone is calculated from the size of the spot.

b) *p-Hydroxyphenobarbitone.* The aliquot of the urine extracted with chloroform is hydrolysed (BUTLER 1956) and extracted with ether. The additional purification is as described for phenobarbitone except that ether is used as organic solvent.

The ether phase is evaporated to 15 ml and extracted with 10 ml borate buffer (pH 10.0) for quantitative spectrophotometric analysis.

If calculation is impossible, paper chromatography is performed as described for phenobarbitone. In the presence of fairly large amounts (> 10 µg/ml urine) the eluate is sufficiently pure for quantitative measurement.

Where smaller amounts are present (10–0.2 µg/ml) the extract is first subjected to paper chromatographic purification (WALKERSTEIN *et al.* 1958) to remove certain phenolic acids

Table 1

Recovery experiments with phenobarbitone and p-hydroxyphenobarbitone. Phenobarbitone and p-hydroxyphenobarbitone added to barbituric-acid-free urine and to barbituric acid-free hydrolysed urine respectively

μg substance added to ml of urine	Conc. $\mu\text{g/ml}$	Phenobarbitone		p-hydroxyphenobarbitone	
		method	recovery %	method	recovery
500 $\mu\text{g}/10$ ml	50		93	b	84
100 $\mu\text{g}/10$ ml	10	b	83	c	60
100 $\mu\text{g}/100$ ml	1	b	58	c	49
100 $\mu\text{g}/500$ ml	0.2	b	57	c	43
25 $\mu\text{g}/500$ ml	0.05	d	ppr 30	d	pp 30
10 $\mu\text{g}/500$ ml	0.02	d	ppr 30	d	ppr 20

- a) Direct spectrophotometric determination.
 b) Spectrophotometric determination after paper chromatography once.
 c) Spectrophotometric determination after paper chromatography twice.
 d) Calculated from the spot size on thin-layer chromatogram

of biological origin. After development the p-hydroxyphenobarbitone (Rf value 0.8-0.9) is located in an aliquot of the extract with diazotized sulphuric acid (HAM & MACKE 1934). p-Hydroxyphenobarbitone, unlike the phenolic acids, reacts weakly with this reagent. After spraying is therefore performed with 0.5 N alcoholic sodium hydroxide to intensify the colour. A non-sprayed aliquot is eluted and re-chromatographed (ALGERI & WALKER 1952). After development and elution, quantitative spectrophotometric measurement is performed.

If the presence of small amounts ($< 0.2 \mu\text{g/ml}$ urine) the eluate is used for two-dimensional quantitative thin-layer chromatography as described for phenobarbitone. Diazotized sulphuric acid and alcoholic sodium hydroxide are used for visualization of the p-hydroxyphenobarbitone.

Recovery test 1) *Phenobarbitone*: Phenobarbitone dissolved in methanol was added to urine from a subject who had taken no barbituric acid compound. The urine samples were examined as stated above. The values given in table 1 are the average values of two determinations.

b) *p-Hydroxyphenobarbitone*: Barbituric-acid-free urine was hydrolysed (BUTLER 1936) and p-hydroxyphenobarbitone dissolved in methanol was added. The urine samples were examined as described above.

As we had only a few milligrams of p-hydroxyphenobarbitone at our disposal at the time of this investigation, the values recorded in table 1 are the result of single determinations.

Results

It is seen in fig. 1 that using a semilogarithmic representation, the plasma level of phenobarbitone fell as a rectilinear function after the administration of the drug had been discontinued. The slope of the curve

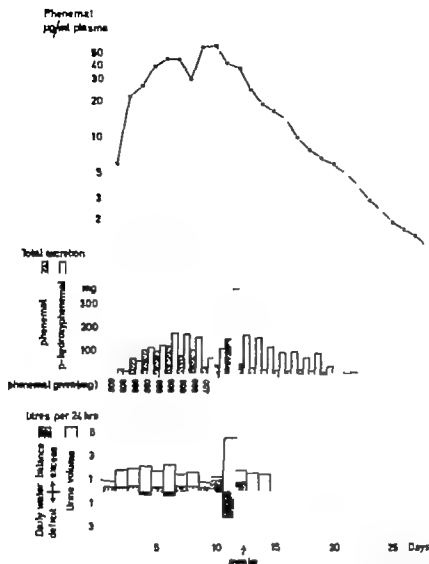


Fig. 1 Relation between the plasma level of phenobarbitone (phenobarbital MFN), the 24-hour excretion of phenobarbitone and p-hydroxyphenobarbitone (p-hydroxyphenobarbital), and the patient's fluid balance.

Phenobarbitone was given by stomach tube in doses of 200 mg each (the last two only of 100 mg each). The doses were given at equal time intervals.

During the anaesthesia the patient received fluid by stomach tube and by intravenous infusion with the aim of obtaining a 24-hour urine volume exceeding 1500 ml.

indicated a 24-hour elimination of 18 per cent phenobarbitone, which gave a biological half-life in plasma of $3\frac{1}{2}$ days.

Phenobarbitone as well as p-hydroxyphenobarbitone were still detect

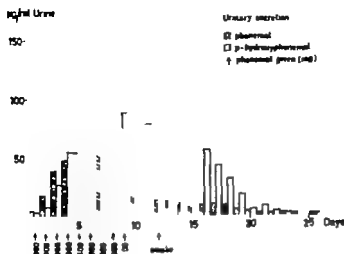


Fig. 2. The amount of phenobarbital and p-hydroxyphenobarbital excreted/ml 24-hour urine.

Same conditions as stated in fig. 1

able in the urine 47 days after the institution of the drug treatment, or 38 days after its discontinuation. Of the 6.4 g phenobarbital given, 3.08 g were recovered within this period. p-Hydroxyphenobarbital constituted 66 per cent of the recovered amount. This corresponds to an excretion of 2.19 g p-hydroxyphenobarbital during this period.

The 24-hour excretions of phenobarbital and of p-hydroxyphenobarbital within the first 28 days after the institution of treatment are shown on fig. 1.

While the excretion of phenobarbital/ml in the urine was maximal on the eighth day after the start of the treatment and then decreased, that of p-hydroxyphenobarbital/ml in the urine reached a maximum on the seventh day thereafter decreasing until the eleventh day (fig. 2). After this another rise set in, so that on the thirteenth day the excretion had almost reached the maximum value. By comparing this variation with the patient's fluid balance (fig. 1), the excretion of p-hydroxyphenobarbital/ml urine was seen to fall in parallel with a reduction of the 24-hour urine volume. When the latter returned to a "normal" level, the amount of p-hydroxyphenobarbital/ml urine increased again. The rise also coincided with the patient's awakening.

In calculating the fluid balance the insensible perspiration was set at 1000 ml per 24 hours. No account was taken of the amount of secretion aspirated daily from the oral cavity. Before treatment the patient was

given anticholinesterases over a long period which stimulated the secretion of saliva. Such a stimulating effect persists for a very long time after discontinuation of the drug treatment. The excess of fluid was thus lower than stated, the amount of aspirated secretion having been up to 100-150 ml per 24 hours.

The amount of p-hydroxyphenobarbitone/ml of urine decreased from the 13th day but did not reach the level of the phenobarbitone excretion till about 10 days later (fig. 2).

During the interval from the 28th to the 47th day after the start of the drug the amounts of both phenobarbitone and p-hydroxyphenobarbitone in the urine fell from 1.0 $\mu\text{g/ml}$ to 0.02 $\mu\text{g/ml}$. These values must be accepted with some reservation, however, as is evident from the results of the recovery tests (table 1).

Discussion

While in previous studies on the human excretion of phenobarbitone (FLEURY & GUINNEBAULT 1918; HALBERKANN & REICHE 1927; LOUS 1954) no more than about 20 per cent of the given dose could be recovered, we succeeded in recovering about 50 per cent by including the amount of p-hydroxyphenobarbitone excreted in the determination.

The excretion of phenobarbitone had not yet ceased 38 days after the last administration, when we had to stop the collection of urine. It is, however, unlikely that about 50 per cent of the given dose still remain accumulated in the organism.

Our analytical method involved certain losses, though not large enough to account for the fairly low recovery.

The investigation, which aimed in the first instance at controlling the patient's condition, did not include examination of faeces. The loss by this route is therefore likewise the losses by other extrarenal excretions e.g. by the aspirated saliva and by sweat not known. As the percentage of the unchanged phenobarbitone recovered in the urine corresponded to that stated by other workers, the absorption from the gastrointestinal tract may be assumed to have been normal.

GLASSON & BENAKIS (1961) hold that in rats only a small proportion of phenobarbitone is excreted in the urine in the form of o-hydroxyphenobarbitone. There has been no evidence to suggest such an excretion in our patient. m-Hydroxyphenobarbitone too was not demonstrable. The non-recovered proportion of the phenobarbitone may nevertheless have been excreted as a metabolite other than p-hydroxyphenobarbitone. An opening of the barbituric acid ring or a dealkylation at the 5-position in the barbituric acid ring are possibilities that must be considered. BENAKIS &

GLASSON (1964) plainly showed that no opening of the ring takes place in rats. Our analytical method has been unable to show however whether this process or a de-alkylation to 5-phenylbarbituric acid or 5-ethylbarbituric acid occurs in man.

Several workers (e.g. REMMER 1959 CONNEY 1960) have shown that phenobarbitone has an inductive effect on an oxidizing enzyme system in the liver. This is also evident from the ratio of excreted amount of phenobarbitone to that of p-hydroxyphenobarbitone during the first few days of the investigation. The fact that the excretion remained at a high level, even though the plasma level of phenobarbitone had fallen to one third of the maximum value supported the theory of an inductive effect.

The fall in the amount of p-hydroxyphenobarbitone/ml of urine between the 7th and the 13th day may be due to a reduced metabolism owing to a failing liver function (KUTT *et al.* 1964)

This hypothesis is in some measure supported by REMMER's (1963) observation that in dogs high plasma levels depressed the oxidative demethylation of metamizol natrium (NFM). To our knowledge the question whether phenobarbitone at high concentrations is also able to depress its own metabolism has not yet been investigated.

Since, however the fall coincided with a diminution of the urine volume, the most obvious explanation seems to be that phenobarbitone, like other anaesthetics (PAPPER *et al.* 1960) depresses the renal function.

Summary

The urinary excretions of phenobarbitone and p-hydroxyphenobarbitone were measured in a patient. The patient was given a total of 6.4 g phenobarbitone by mouth, distributed over a period of 9 days.

Examination of the plasma after discontinuation of the treatment revealed a biological half-life in the plasma of 3½ days.

Both phenobarbitone and p-hydroxyphenobarbitone were detectable in the urine as late as 38 days after the last dose had been given. During this period about 50 per cent of the total amount of phenobarbitone given was recovered in the urine, of which about two-thirds was in the form of p-hydroxyphenobarbitone.

The analytical technique used excluded interference by other substances, but proved unsuitable for demonstrating whether the non-recovered proportion of phenobarbitone had been excreted as a compound in which an opening had occurred of the barbituric acid ring, or whether it had been excreted in the form of 5-phenylbarbituric acid or 5-ethylbarbituric acid. Neither o-hydroxyphenobarbitone nor m-hydroxyphenobarbitone was detected in the urine.

Acknowledgement

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Some Aspects of Clopenthixol Metabolism in Rats and Humans

By

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With the introduction of chlorpromazine in psychiatry phenothiazine derivatives have played an important role in psychopharmacology. Since 1958 the thioxanthenes have also gained considerable attention for their use as neuroleptic agents (PETERSEN & MØLLER-NIELSEN 1964). Work has been initiated to study the metabolism of these drugs. Previous investigations were carried out on chlorprothixene (ALLGÉN *et al.* 1960; HUIS & KHAN 1967; RAAFLAUB 1967). The present report describes some aspects of clopenthixol (sordimol B), 2-chloro-9-(3-(4-(2-hydroxyethyl)-1-piperazinyl)-propylidene)-thioxanthene (CPX) metabolism in rat and man, which suggest some difference in the metabolic pathway as compared with that of other phenothiazines.

Experimental

Collection of urine samples

Two groups of 10 male Wistar rats (180-220 g) were used in this test. Control urine was collected before treatment. A dose of 25 mg/kg of CPX was given i.p. the morning. The urine and faeces excreted over 24 hrs were collected separately and then pooled and deep frozen till analysed. In another experiment a dose of 25 mg/kg i.p. was given every morning for 4 days. Samples of urine and faeces excreted over 24 hrs were collected separately up to two days after the last dose and deep frozen. The samples were pooled before analysis.

For the human study three patients on a daily dose of 25, 35 and 50 mg respectively of clopenthixol were selected. Before collecting the urine samples, any other drug in use was withdrawn for 5 days. Samples of urine excreted over 24 hours were then collected and analysed.

Extraction of urine from rats.

Non-confused fraction.

50 ml of urine was adjusted to pH 9 with 1.0 N-NaOH and extracted three times with an equal volume of 1,2-dichloroethane (DCE). The contents were shaken mechanically

for 20 minutes in a separating funnel. The organic phase was separated by centrifugation. The organic extract was washed once with an equal volume of ammonia buffer pH 9 dried over sodium sulphate and filtered. The filtrate was evaporated to a volume of about 5 ml in rotary evaporator under vacuum at 40°. Final drying of the extract was done in centrifuge-tube on steam bath under a stream of nitrogen. The residue was dissolved in 500 µl of DCE. 20 to 50 µl was spotted on the plate.

Conjugated fraction.

Acid hydrolysis

After the exhaustive extraction of the non-conjugated metabolites the residual urine was divided into two equal portions. To one portion concentrated hydrochloric acid equal to $\frac{1}{2}$ of the volume of the urine was added. Both portions were heated on a steam bath for 30 minutes. The urine was adjusted to pH 9 and extracted with three equal volumes of DCE. The organic extract was prepared for chromatography as described above. The residue was dissolved in 250 µl of DCE.

Hydrolysis with β -glucuronidase

After the exhaustive extraction of the non-conjugated metabolites the residual urine was adjusted to pH 5.5 with 1.0 N HCl. Phosphate buffer pH 5.5 was added in amounts equal to $\frac{1}{2}$ of the volume of the urine, which was then divided into two equal portions. To one portion β -glucuronidase (bacterial/Eligma Chemical Co.) was added to give a concentration of 500 units/ml of urine. The samples were incubated at 37° for 18 hours and then extracted with three equal volumes of DCE after adjustment to pH 9 with 2 N NaOH. The organic extract was prepared for chromatography in the usual way. The residue was dissolved in 250 µl of DCE. In another trial the urine was hydrolyzed directly without previous extraction of the non-conjugated metabolites. 50 ml of urine was treated as mentioned above under hydrolysis with β -glucuronidase. Combined hydrolysis with β -glucuronidase and sulphatase was also performed using a concentration of 500 and 250 units per ml of urine respectively.

Extraction of faeces from rats.

2 g of faeces were homogenized with 20 ml of 0.1 N-HCl in Searvall Omni Mixer for three minutes. The homogenate was adjusted to pH 9 with 1 N-NaOH and transferred into a separating funnel. The extraction was carried out with three equal volumes of DCE. The organic extract was washed once with an equal volume of ammonia buffer pH 9 and prepared for chromatography as mentioned above under the extraction of urine. The residue was dissolved in 1 ml of DCE.

Extraction of human urine

The method for the analysis of human urine was the same as for the urine from rats. In view of the small dose administered and very low concentration of the metabolites present, the urine samples were concentrated in either of the following two ways.

1. Applejack concentration method (FORREST *et al.* 1966). In this method 1000 ml of urine was deep frozen in a 1 litre plastic bottle. It was transferred to refrigerator maintained at -4-5° and inverted to collect the slowly melting urine. The first 50 ml of collected urine was analysed in the usual way.
2. Extraction with ion-exchange resin (DOLZ *et al.* 1966). These authors have mentioned the use of Amberlite ion-exchange resin paper for the extraction of phenothiazines and their metabolites from human urine. In this process Amberlite ion-exchange resin CG 120

(IR 120 in Na⁺ form) was used instead of paper loaded with resins. 200 ml of urine was adjusted to pH 5.9 with 0.1 N HCl. Amberlite ion-exchange resin (5-6 g) was then added. The mixture was shaken intermittently for 30 minutes. The contents were centrifuged and the resin was transferred to a separating funnel. The extraction was done with a 100 ml mixture of borax buffer pH 9.3 and DCE (equal volume). The organic phase was separated and the aqueous phase was extracted with another 30 ml of DCE. The organic extracts were mixed together and prepared for chromatography.

Model system for aromatic ring hydroxylation.

Ring hydroxylation was tried using a modified model system suggested by COCCIA & WARTENBERG (1967). 10 μ mol of CPX were incubated in air at 37°C for 20 hours and the reaction mixture was then extracted three times with 25 ml of DCE at pH 9 and prepared for chromatography.

Thin-layer Chromatography (TLC).

Preparation of plates

20 x 70 cm plates were coated with a 250 μ thick layer of silica gel acc. to Stahl (Merck) and activated at 110°C for 30 minutes. 20-30 μ l of the extract was spotted on the plate along with any available authentic metabolites. The chromatograms were developed in the following four different solvent systems:

1. Acetone n-heptane DEA (60:40:10)
2. Benzene DMF DEA (80:10:10)
3. Butanol acetic acid water (80:20:20)
4. Ether DEA (90:10)

DEA = diethylamine

DMF = dimethylformamide.

The tank was equilibrated for 30 minutes before use. Approximate length of run was 15 cm.

Visualization of spots

The following reagents were used to visualize and identify the spots:

1. A mixture of concentrated sulphuric acid and formaline (48:2) was sprayed on the plate and the spots were located under UV light.
2. Dragendorff's reagent (STRAHL 1967).
3. Persulphate reagent (SMITH 1963).
4. Sodium nitroprusside-acetaldehyde (STAHL 1967).
5. Ferric chloride for detection of phenols (STAHL 1967).
6. Ehrlich's reagent (Diaz-reagent) (STAHL 1967).
7. Gibbs reagent (STAHL 1967).
8. Folin-Ciocalteu reagent for phenols (STAHL 1967).

UV absorption curves.

The urinary extract was dissolved in 1.0 ml of DCE. Of this 0.5 ml was spotted on the plate in the form of a line 10 cm in length. The chromatogram was developed in solvent system No. 2 and thoroughly dried. A portion of the chromatogram was sprayed with a mixture of sulphuric acid-formaline. Corresponding bands of metabolites were eluted in 0.1 N HCl, filtered through a G-4 glass filter and the UV curve was drawn on a Beckman DB-G spectrophotometer.

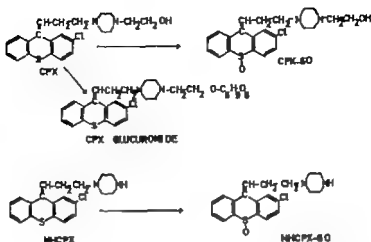


Fig. 1 Structure of identified metabolites. Clopenthixol (CPX); clopenthixol-glucuronide (CPX-glucuronide) clopenthixol-sulphoxide (CPX SO) 2-chloro-9-(3-(1-piperazinyl)propylidene)thioxanthene (NHCPX) and 2-chloro-9-(3-(1-piperazinyl)propylidene)thioxanthene-sulphoxide (NHCPX-SO).

Derivative formation.

Oxidation of CPX to CPX SO was carried out by the method suggested by Koroed *et al.* (1966) on TLC plates and the chromatogram was developed in solvent systems 1 and 2.

Standards.

The standard substances available i.e. clopenthixol (CPX), clopenthixol-sulfoxide (CPX SO), 2-chloro-9-(3-(1-piperazinyl)propylidene)-thioxanthene (NHCPX) and 2-chloro-9-(3-(1-piperazinyl)propylidene)-thioxanthene-sulfoxide (NHCPX-SO) were added to the urine and then extracted as mentioned under extraction of urine and used as reference standards (for formulae see fig. 1)

Results

Clopenthixol metabolism in rats

A spot corresponding in R_f -value to standard CPX appeared on the chromatogram (fig. 2 and table 1) This spot (No 1) showed a colour reaction with a mixture of H_2SO_4 formaline (yellow fluorescence in UV light) and with persulphate reagent (orange fluorescence in UV) identical to standard CPX. The spot gave no colour reaction with reagents used to detect the phenolic metabolites. This indicated the absence of a hydroxyl group in the ring system. For further confirmation the spot was oxidized to its sulphoxide with ethanolic hydrogen peroxide as suggested by Koroed *et al.* (1966) and chromatographed with authentic CPX SO

Table I

Approximate R_F -values of metabolites in the non-conjugated and conjugated fractions.

No	Solvent system	Solvent system	Solvent system	Solvent system
	1	2	3	4
1	.57	.81	.35	.49
2	.41	.69	.19	.23
3	.26	.43	.42	-
4	.17	.31	.25	.08
5	.07	.18	.09	-
Standards				
CPX	.57	.81	.35	.49
CPX-SO	.41	.70	.19	.23
NHCPX	.26	.43	.42	-
NHCPX SO	.18	.31	.25	.08

synthesized in our laboratories. The oxidation product showed a colour reaction with persulphate reagent (green fluorescence in UV) and an R_F -value identical to authentic CPX-SO. Approximate quantification

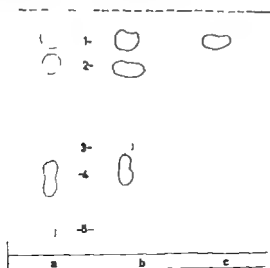


Fig. 2. (a) chromatogram of extract of rat's urine (non conjugated fraction). (b-c) chromatograms of extracts of human urine, non-conjugated and conjugated fraction respectively. Solvent system No. 2.

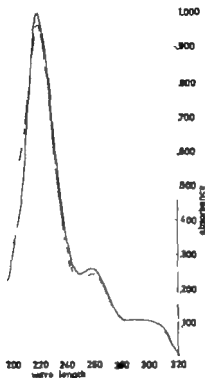


Fig. 3. UV absorption curve for authentic clopenthixol-sulphoxide (CPX-SO) (-----).
UV absorption curve for spot No. 2 (CPX-SO) (—)

made by comparing the intensity of the spot with graded standards, indicated that it did not exceed 2–3 $\mu\text{g/ml}$ of urine.

Spot No. 2 (fig. 2) showed characteristics identical to clopenthixol sulphoxide (CPX-SO) as far as the R_f -values in different solvent systems (table 1), colour reactions with H_2SO_4 , formaline and persulfate reagent (yellow and green fluorescence in UV respectively) were concerned. This spot also showed a negative colour reaction with the reagents used to detect phenols. This indicated that the metabolite was simply a sulphoxide of CPX. For further identification spot No. 2 was eluted in 0.1 N HCl and its UV absorption curve was compared with authentic CPX-SO. Both the curves showed maximum absorption at 220 nm and 258 nm (fig. 3) whereas CPX gives a maximum at 230 and 268 nm. The approximate quantity of CPX-SO was 15–20 $\mu\text{g/ml}$ of urine.

In view of previous experience with flupenthixol (JORGENSEN *et al.* 1969), degradation of side chain to form NHCPX or NHCPX-SO was expected when a large spot (No. 4 fig. 2) appeared on the chromatogram. This

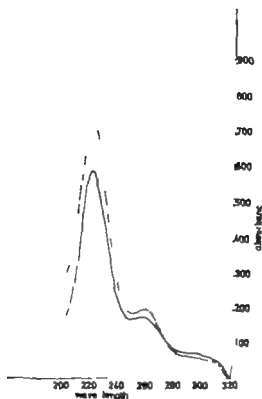


Fig. 4 UV absorption curve for authentic NHCPX-SO (-----).
UV absorption curve for spot No. 4 NHCPX-SO (—).

spot showed R_f -values (table 1) and colour reactions with H_2SO_4 , formaline and persulphate reagent (yellow and green fluorescence respectively in UV) identical to the authentic NHCPX-SO synthesized in our laboratories. The metabolite was identified as NHCPX-SO when its UV-absorption curve was also found to be identical to authentic NHCPX-SO (fig. 4). The approximate quantity of NHCPX-SO present was 15–20 $\mu g/ml$ of urine. Spot No. 3 (fig. 2) showed yellow fluorescence with a mixture of H_2SO_4 , formaline and orange fluorescence with persulphate reagent in UV-light. Its R_f -values were identical to authentic NHCPX in four different solvent systems (table 1) but the quantity was too small to carry out any further confirmatory tests as mentioned above. On these grounds the metabolite was assumed to be NHCPX. This spot appeared only in the urine samples of rats given repeated doses.

Spot No. 5 (fig. 2) remained unidentified. This metabolite showed reddish pink fluorescence with a mixture of H_2SO_4 , formaline in UV and also gave an orange colour with Dragendorff's reagent. This spot gave no

colour reaction with phenolic reagents. The conjugated fraction of rats urine gave no evidence that any of the identified metabolites were excreted in a conjugated form. No phenolic metabolite could be detected in this fraction.

Analysis of faeces from rats showed the presence of the parent substance and the 4 above mentioned metabolites. Intact CPX and NHCPX were present in comparatively larger quantities than in urine whereas only traces of their sulphoxides were detected in the faeces.

Clopenthixol metabolism in man

The following results were obtained by the analysis of urine from the patient on a 50 mg dose. In the other two cases the concentrations of the metabolites were found to be very small owing to the very low dosage.

Non-conjugated fraction

Four spots appeared on the chromatogram (fig. 2). Their R_f -values in four different solvent systems and colour reactions with different colouring reagents were identical to standards, CPX, CPX-SO, NHCPX and NHCPX-SO. These spots also corresponded to spot No. 1, 2, 3 and 4 detected in the urine of rats. No further efforts were made to confirm the identity of these spots as the quantities of the metabolites were too small to allow spectrophotometric studies.

Conjugated fraction

Hydrolysis of the residual urine with β -glucuronidase after the exhaustive extraction of the non-conjugated metabolites showed that CPX is also excreted in a conjugated form with glucuronic acid. No other metabolites, as for instance phenolic compounds, were detected in the conjugated fraction.

Discussion

The metabolism of CPX appeared to be similar in the rat and man. Only small amounts of CPX have been found to be excreted in a conjugated form with glucuronic acid in man and none in rats in spite of the fact that CPX and three other identified metabolites all possess glucurogenic groups. According to BRAY (1953) compounds containing glucurogenic groups do not necessarily form glucuronides although they might be expected to do so.

Numerous reports on phenothiazines and other aromatic compounds indicate that hydroxylation in the ring structure is an important route

of metabolism. Hydroxylated metabolites are generally excreted as glucuronide conjugates. Thus a number of phenolic metabolites of chlorpromazine have been identified (FISHMAN & GOLDENBERG 1965). No phenolic metabolites of CPX have been detected in the present studies. This might indicate that the thioxanthene nucleus is not readily hydroxylated *in vivo*. To what extent such a difference in the heterocyclic ring may affect the hydroxylation is not known. WILLIAMS (1959) stated that in a ring structure like phenothiazine, the heterocyclic nitrogen plays some role in the orientation of the hydroxyl group in the attached benzene rings. Whether it does play any role in facilitating the hydroxylation itself of the phenothiazine is not known. The exact characteristics of the hydroxy-derivatives of clopenthixol are not known since attempts to synthesize these compounds have failed. Our conclusions are based on the negative colour reaction with the phenolic reagents. Attempts to introduce hydroxylation of clopenthixol *in vitro* by a modified system (COCCIA & WESTERFELD 1967) were also unsuccessful, suggesting that the thioxanthene ring of CPX is difficult to hydroxylate, whereas hydroxylation of chlorpromazine has been successfully done by the same modified model system. Although failure to induce hydroxylation in *in vitro* systems is no guarantee that hydroxylation will not take place in the animal or human body the present results do indicate that hydroxylation of the thioxanthene nucleus does not readily occur.

The unidentified metabolite also showed a negative colour reaction with phenolic reagents. N-oxide formation might be a possibility and such N-oxides have been identified in chlorprothixene and chlorcyclizine (RAAFLAUB 1967 KUNTZMAN 1967).

In the faeces of rats CPX and NHCPX were found in large quantities as compared to urine. The excretion pattern observed showed that non-sulphoxide i.e. CPX and NHCPX were mainly excreted in the faeces with only traces of their sulphoxides, whereas sulphoxides i.e. CPX-SO and NHCPX-SO were mainly excreted in the urine with only traces of CPX and NHCPX. The mechanism of such an excretion pattern is not well understood. Enterohepatic circulation of the drug and its elimination in the faeces may be one of the factors involved, (LEWIS 1962). The excretion of large quantities of CPX in the faeces as compared to that in urine might be due to the side-chain as pointed out by FLANAGAN *et al* (1962).

Summary

Chromatographic techniques have been used to analyze urine and faeces from rats and urine from man to elucidate the metabolism of

clopenthixol (CPX). The identification of clopenthixol-sulfoxide, NH-clopenthixol and NH-clopenthixol-sulphoxide is reported (fig. 1). A fourth metabolite found in the urine from rats remained unidentified. In man CPX has been found to be excreted in a conjugated form with glucuronic acid which was not the case in rats. Hydroxylation of the thioxanthene ring has not been observed either in man or in the rat, nor has it been possible to induce hydroxylation in *in vitro* systems.

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The Sensitivity of Adrenergic Excitatory and Inhibitory Receptors in the Smooth Muscle of the Rabbit Urinary Bladder

By

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In a previous investigation (EDVARDSEN & SETEKLEV 1968) it was found that muscle strips isolated from the dome of the urinary bladder of cats and rabbits relaxed after the administration of adrenaline, noradrenaline and isoprenaline, whereas a contraction was elicited by adrenaline and noradrenaline in muscle taken from the bladder base. The contraction had a shorter latency than the inhibition, which could explain the biphasic response to electrical stimulation of the hypogastric nerve. This response consists of an initial contraction followed by an inhibition of the rhythmic contractions and tone, resulting in closure of the "sphincter" and relaxation of the corpus. However the detrusor muscle was found to contain not only inhibitory but also excitatory adrenergic receptors. The latter became evident when a β -adrenergic blocking agent had been given. On the other hand, inhibitory responses were usually recorded in muscle strips from the bladder base when the excitatory response had been abolished by α -adrenergic blocking agents. The smooth muscle of the urinary bladder thus appears to contain excitatory as well as inhibitory adrenergic receptors. The response elicited by the neurotransmitter - i.e. either contraction or inhibition - must therefore depend on the sensitivity of the two receptors to the transmitter.

The aim of the present investigation was to study the sensitivity of the adrenergic receptors in the urinary bladder base and dome by determining

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the response to increasing doses of adrenaline, noradrenaline and isoprenaline before and after pharmacological blocking of the receptors.

Under certain conditions the smooth muscles of the urinary bladder are presumably influenced by the adrenergic and the cholinergic neurotransmitters simultaneously. Therefore, a study of the muscular response to acetylcholine and the effect of the cholinergic transmitter on the adrenergic receptors was also included in the present investigation.

Material and Methods

In the experiments 23 rabbits were used. The animals were killed by an air embolus and the urinary bladder removed as quickly as possible, emptied and submerged in Krebs solution at room temperature.

Muscle strips, about 1 cm long and 0.5 mm thick, were dissected from the urinary bladder. One strip was taken from the bladder base (below the ureters), another from the fundus. The strips were mounted in the same organ bath containing modified Krebs solution (Na^+ 136.9 K 3.9 Ca^{2+} 2.5 Mg^{2+} 1.2, HCO_3^- 15.3, H_2PO_4^- 1.2, Cl^- 133.6 and glucose 11.5 mM) of 36 ml, aerated with gas mixture of 95% O_2 and 5% CO_2 . The preparations were allowed to equilibrate for about two hours before commencing the experiments.

The mechanical activity was recorded isometrically either by mechano-electric transducer valves (RCA 5437) connected to a Hewlett Packard recorder or by Grass Transducers (Model FT 03) connected to a Grass Polygraph.

Adrenergic and adrenergic blocking agents. The excitatory as well as the inhibitory adrenergic receptors were stimulated by (–)-adrenaline D-bitartrate and (–)-noradrenaline D-bitartrate and the inhibitory adrenergic receptors by (±)-isoprenaline sulphate. The agents were added to the organ bath so as to bring the concentrations in the bathing solution from 1.8×10^{-10} to 3×10^{-7} M. The concentrations refer to the base. To ensure a maximal response, the preparations were exposed to the drugs for 4 minutes and a recovery time of at least 6 minutes was allowed before a further dose was added. Phenoxybenzamine and phentolamine (regitline ® Ciba) were used in order to block the excitatory (α) and propranolol (Inderal ® ICI) to block the inhibitory (β) receptors. The experiments were carried out after exposure to the blocking agents for least 15 min.

Cholinergic and cholinergic blocking agents. Carbachol (carbacholimus NFN) chloride in concentrations from 5.5×10^{-10} – 5.5×10^{-7} M was used to obtain long lasting cholinergic stimulation and atropine sulphate (1.4×10^{-6} M) to block this effect.

Results

The bladder base – Fig. 1 shows a graph of the response to increasing doses of adrenergic drugs in a preparation taken from the base of a rabbit urinary bladder. Adrenaline in doses from 3×10^{-8} to 3×10^{-7} M elicited an inhibition of the rhythmic contractions in this preparation, but in doses at or higher than 1.5×10^{-6} M the response was reversed and a contraction was obtained. By increasing the dose, the excitatory response

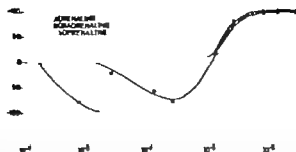


Fig. 1. The inhibitory (below zero) and excitatory effect (above zero) obtained on a muscle strip from the urinary bladder base with increasing doses of adrenaline (●), noradrenaline (○) and isoprenaline (□) calculated in per cent of the maximal response.

reached a maximum at 1.5×10^{-5} M. Fig. 2 illustrates this reversal obtained by increasing the dose of adrenaline. A concentration of adrenaline of 3×10^{-7} M produced an inhibition whereas 1.5×10^{-6} M elicited a contraction although an inhibition of the rhythmic activity after the washout was also clearly seen, indicating a mixed response. In the preparation from which the results in fig. 1 are taken, small doses of *noradrenaline* did not inhibit the usual mechanical activity found in other preparations, but doses at or above 1.5×10^{-6} M evoked contractions. Isoprenaline depressed the spontaneous contractions in concentrations of 9×10^{-9} M and complete inhibition was obtained with a concentration of 1.8×10^{-8} M.

For all preparations used, the threshold concentration necessary for an inhibitory response were lower than the threshold concentrations for the excitatory one. The threshold concentration varied from preparation to pre-

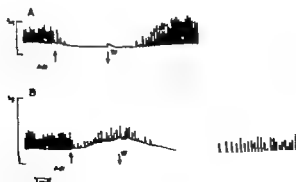


Fig. 2. Reversal of the response to adrenaline in longitudinal muscle strip from the bladder base when the dose is increased from 3×10^{-7} M (A) to 1.5×10^{-6} M (B). Arrows indicate when the agent was given. W: washout.

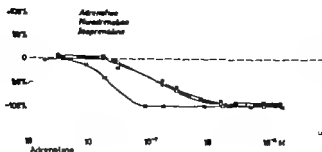


Fig. 3 The inhibitory response of muscle strip from the dome of the urinary bladder to increasing doses of adrenaline (●), noradrenaline (○) and isoprenaline (□)

paration. In 9 experiments the average threshold concentration for inhibitory responses to noradrenaline was 3.3×10^{-7} M and varied between 3×10^{-9} M and 1.5×10^{-6} M for adrenaline 7.8×10^{-8} (25 experiments, range 3×10^{-9} to 3×10^{-7} M) and for isoprenaline 5.8×10^{-9} (9 experiments, range 1.8×10^{-9} to 9×10^{-9} M).

The excitatory effects of adrenaline and noradrenaline were elicited in 22 out of 32 preparations. In these 22 experiments the average threshold concentration for adrenaline was 10^{-6} (range 3×10^{-8} to 3×10^{-6} M and for noradrenaline 2×10^{-6} (9 experiments, range 1.5×10^{-7} – 1.5×10^{-5} M).

The bladder fundus – In muscle strips taken from the bladder fundus, adrenaline as well as noradrenaline and isoprenaline produced an inhibition of the spontaneous rhythmic activity and tone (fig. 3). The preparations were found to be most sensitive to isoprenaline (average threshold 1.8×10^{-8} M 13 experiments, range 1.8×10^{-9} – 9×10^{-8} M) and adrenaline (average threshold 3×10^{-8} M 31 experiments, range 3×10^{-9} – 3×10^{-7} M).

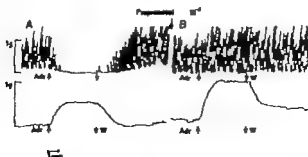


Fig. 4 Effect of adrenaline (1.5×10^{-6} M) on a preparation obtained from the bladder fundus (above) and base (below) before (A) and after (B) propranolol (1.4×10^{-6} M). The inhibitory response in the fundal preparation is blocked and the magnitude of the excitatory response in the bladder base preparation is increased.

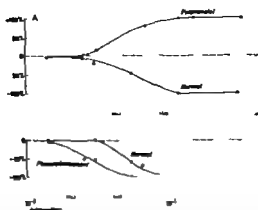


Fig. 5 A The reversal of the response in preparation from bladder fundus. Effect of increasing doses of adrenaline in the normal preparation (●) and (○) after treatment with propranolol (3.4×10^{-6} M) B phenoxybenzamine (2.9×10^{-6} M) increases the sensitivity of the inhibitory response to adrenaline in fundal preparation. Normal preparation ● phenoxybenzamine treated ○.

and less sensitive to noradrenaline (average threshold 2.1×10^{-7} M, 10 experiments, range 3×10^{-9} to 1.5×10^{-6} M). High concentrations of adrenaline and noradrenaline (3×10^{-6} and 1.5×10^{-5} M) produced a contraction in 2 out of 33 preparations in these series of experiments.

Effect of β -adrenergic blocking agent – Propranolol (Inderal ® ICT) added to the bathing solution to make a concentration of 3.4×10^{-6} M was used to block the inhibitory β -receptors (EDVARDSEN & SETEKLEV 1968).

In preparations isolated from the *bladder base* the sensitivity of the excitatory response to adrenaline was increased in 6 out of 8 preparations, after the β -receptors had been blocked by propranolol (figs. 4 & 6) thus demonstrating the release of an inhibitory effect on the muscle.

Propranolol always blocked the inhibitory response to adrenaline and noradrenaline in preparations taken from the *fundus* of the urinary bladder and in 10 out of 13 preparations, the responses were reversed and contractions elicited. In the experiment shown in fig. 5A the threshold of the excitatory and the inhibitory response previous to the blockade was the same. Similar results regarding the threshold doses were encountered in 3 other experiments while in 4 experiments, the threshold dose for the excitatory response was higher (5–10 times) and 5–10 times lower in 3 experiments.

In 10 experiments the average threshold concentration of adrenaline necessary to elicit excitatory responses was 7.8×10^{-7} M (range 3×10^{-9} –

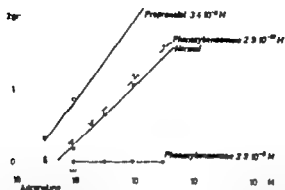


Fig. 6 Amplitude of the sustained contraction in response to increasing doses of adrenaline in a normal preparation (●) after treatment with propranolol 3.4×10^{-6} M (○), a low dose of phenoxybenzamine (2.9×10^{-10} M) (+) and a 10 times higher dose of phenoxybenzamine (●). Propranolol increases the sensitivity of the excitatory response while phenoxybenzamine depresses it.

3×10^{-6} M) i.e. about the same as the average threshold concentration for the excitatory response in tissue from the bladder base.

Effect of α -adrenergic blocking agent – Phenoxybenzamine and phentolamine (regitine ® Ciba) have been shown to abolish the excitatory response of the urinary bladder to adrenaline and to noradrenaline (EDVARDSEN & SETEKLEIV 1968). Similar results were obtained in the present series of experiments. Fig. 6 shows that a concentration of 2.9×10^{-10} M of phenoxybenzamine in the bathing solution reduced the excitatory response to adrenaline and that the response was completely blocked by 2.9×10^{-9} M. In 6 out of 11 experiments phenoxybenzamine increased the sensitivity of the preparation to the inhibitory effect of adrenaline and noradrenaline (fig. 5B) which shows that this effect is opposed by the excitatory component of the adrenaline effect in the bladder.

Effect of cholinergic stimulation on the response to adrenaline – Since an increased activity is found in the hypogastric nerve during micturition (when a decreased activity is expected) it has been suggested that liberation of the cholinergic transmitter might block the inhibitory effect of the adrenergic transmitter on the bladder corpus or in another way unmask the excitatory effect (EDVARDSEN, personal communication). It was therefore of interest to determine whether cholinergic stimulation could influence the adrenergic response.

In order to obtain a constant cholinergic stimulation of the muscles, carbachol was added to the bathing solution. In fundal strips as well as in muscle strips obtained from the base, a concentration of carbachol of

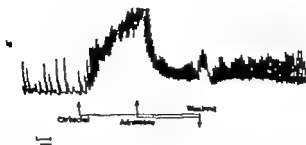


Fig. 7 The opposite effect of carbachol and adrenaline on the urinary bladder. Carbachol (2.8×10^{-8} M) increases the spontaneous activity and tension. Adrenaline (9×10^{-7} M) given when the effect of carbachol is maximal inhibits the mechanical tension and activity

2.8×10^{-8} to 2.8×10^{-7} M increased the spontaneous rhythmic activity and tone of the preparations without producing a tetanic contraction (fig. 7). When adrenaline (9×10^{-7} M) was given in addition, a response qualitatively similar to that found in the untreated preparation was obtained, resulting in an inhibition of the rhythmic contractions and tone (fig. 7). A reversal of the inhibitory response to adrenaline in bladder fundal strips was never recorded. On the contrary when the effect of increasing doses of adrenaline was measured before and after carbachol (2.8×10^{-8} M in this experiment) the preparations were about 100 times more sensitive to the inhibitory effect of adrenaline (fig. 8A). Carbachol thus potentiates the inhibitory action of adrenaline. In a muscle strip from the bladder base where adrenaline elicited contractions of the



Fig. 8. A. Effect of carbachol (2.8×10^{-8} M) on the inhibitory response of a fundal strip to increasing doses of adrenaline. The sensitivity is increased. B. Muscle preparation from the bladder base. Carbachol (2.8×10^{-8} M) reverses the effect of smaller doses of adrenaline. \square reduces the effect of higher doses of adrenaline.

muscle carbachol caused a different response. In these preparations carbachol reversed the response to lower doses of adrenaline and reduced the sensitivity (increased the threshold) and the magnitude of the excitatory response to higher doses of adrenaline (fig. 8B). Blocking of the α -adrenergic stimulation in the fundal strip with phenoxybenzamine (2.9×10^{-6} M) or the β -adrenergic component with propranolol (3.4×10^{-6} M) in the strips from the bladder base, did not alter the sensitivity of the responses to adrenaline in the carbachol-treated preparations.

When adrenaline was given first, the threshold for the excitatory effect of carbachol was increased in fundal strips (where adrenaline caused an inhibition) as well as in preparations from the bladder base (where adrenaline elicited a contraction).

These results were confirmed in 16 preparations from 3 different bladders.

Discussion

The present investigation has disclosed that the responses of the corpus and the base of the urinary bladder to drugs which act both on adrenergic α - and β -receptors depend firstly on the magnitude of the dose given. The inhibitory response is most sensitive and small doses of adrenaline produce an inhibition only while higher doses are necessary to stimulate the excitatory receptors. Secondly since tissue from the bladder fundus as well as from the base contains both types of receptors, the response which appears is determined by the relative size of each of the two opposing components involved. In the fundus the inhibitory dominates over the excitatory response. In tissues from the bladder base, inhibition is elicited by small doses of adrenaline, but the inhibition usually is overcome by the excitatory response when higher doses of adrenaline and noradrenaline are used. In the base, therefore, the excitatory response is quantitatively the stronger.

In trying to explain the variation between the response to adrenergic drugs of these two types of tissue some anatomical differences have to be taken into account.

The smooth muscle of the bladder base, including the so-called internal sphincter and the trigone area (musculus trigonalis) appears to have been developed from the mesoderm while the detrusor muscle is derived from the endoderm. The difference in origin might indicate different functional properties.

The difference in sensitivity between the inhibitory and the excitatory receptors to adrenergic drugs might in some way be correlated to the density of the adrenergic fibres to the muscles in these two regions. In the

base of the cat's urinary bladder HAMBERGER & NORBERG (1965) found a relative rich adrenergic innervation whereas few adrenergic nerves were in contact with the detrusor muscle cells. The same seems to hold true for the rabbit bladder (NORGRÉN & SETTEKLEV unpublished). The smooth muscle of the bladder dome is therefore probably exposed to much less of the adrenergic transmitter liberated from the hypogastric nerve than the sphincter muscles. On the other hand, the average threshold dose for the excitatory response elicited after the inhibitory response had been blocked in the fundal muscle, was about the same as the threshold dose in tissue from the bladder base.

The dose necessary to activate the excitatory receptor *in vitro* is high, so high in fact that one has to take into consideration whether any physiological significance can be attributed to it.

However a contraction of the bladder outlet can also be obtained by hypogastric nerve stimulation (LEARMONTH 1931 GRÖNE 1965 EDVARDSEN 1967). Sufficient transmitter must therefore be released during such stimulation to excite the receptors. The high dose of adrenergic drugs necessary could also be due to inaccessibility of the receptors caused by diffusion barriers or to other factors which prevent the drugs reaching the receptors. A comparison might be made with the failure of atropine to block the cholinergic transmitter released by electrical stimulation of the pelvic nerves, whereas the effect of circulating acetylcholine is readily abolished (URSILLO & CLARK 1956).

The different response patterns in the bladder base and fundus could also be due to different properties of the smooth muscle cells. The different spontaneous rhythmic activity of the preparations from these two regions (EDVARDSEN & SETTEKLEV 1968) indicates variations in the physiological properties of the cells. The sphincter muscle is usually quiet whereas the detrusor muscle exhibits vigorous rhythmic activity. Based on the knowledge from other types of smooth muscle one would expect the detrusor muscle to have a low unstable membrane potential which is hyperpolarized by adrenaline and the sphincter muscle cells to have a higher and more stable membrane potential, which is depolarized by adrenaline. Preliminary studies of the electrical activity of the urinary bladder seem to confirm these assumptions.

The functional significance of the adrenergic innervation of the urinary bladder has been disputed. It closes the bladder outlet during ejaculation and also when urine is collected in the bladder (BARRINGTON 1915 SACKS 1966 GRÖNE 1965 EDVARDSEN 1967). In addition importance has been attributed to a reflex relaxation of the bladder initiated by stretch receptors in the bladder wall and mediated via the pelvic nerves to the spinal cord where adrenergic fibres in the hypogastric nerves constitute

the efferent link (EDVARDSEN 1967). With regard to the latter function, the results of recent anatomical studies are not in favour of an adrenergic effect directly on the detrusor muscle, since the adrenergic innervation to the muscle is so scanty in this region (HAMBERGER & NORBERG 1965). The present investigation has, however, not only demonstrated adrenergic inhibitory receptors in this type of smooth muscle, but also that they are sensitive to small doses of adrenergic drugs. That the bladder relaxes in response to hypogastric nerve stimulation has also been demonstrated by several investigators (For ref. see EDVARDSEN 1967). However the anatomical evidence necessitates that nervous mechanisms other than a direct action on the smooth muscle have to be taken into consideration.

Another question which recent studies have raised is the interaction between the parasympathetic and the sympathetic nervous system during micturition. The present findings that cholinergic stimulation increases the sensitivity of the adrenergic inhibitory response is not consistent with the hypothesis that release of the cholinergic transmitter induces a reversal of the adrenaline effect due to blocking of the inhibitory receptors, or an unmasking of the excitatory ones. The increase observed in the efferent electrical activity in the hypogastric nerve during micturition can therefore hardly be explained in this way.

The findings indicate that the membrane potential is of importance for the effect elicited. Cholinergic drugs depolarize the smooth muscle and so does stretching of urinary bladder smooth muscle (BURNSTOCK & PROSSER 1960). Since depolarization renders the bladder more sensitive to the adrenergic transmitter this effect should produce more favourable conditions for the inhibitory reflex mediated by the hypogastric nerve during the collecting phase.

In preparations which respond by a contraction to adrenaline, cholinergic stimulation reverses the effect to lower doses of adrenaline and decreases the excitatory response to higher doses. This action would thus promote a relaxation of the sphincter region during micturition.

With regard to the participation of the parasympathetic nerves in the opening of the bladder outlet during micturition, it has been claimed that they have a slight relaxing effect on the sphincter (ELLIOT 1907; LEAR MONTH 1931). LANGWORTHY & MURPHY (1939) however could not demonstrate cholinergic nerves in the region of the internal sphincter and the trigone area, so that the opening of the bladder outlet has been attributed to detrusor muscle contraction which, because of the anatomical arrangement of the smooth muscles around the bladder neck, opens the urethral orifice (RUCH 1960). At present, therefore, it is difficult to attribute any physiological significance to the inhibitory cholinergic effect on adrenergic excitatory responses in the urinary bladder.

Summary

The responses of muscle strips from rabbit urinary bladders to increasing doses of adrenergic agents have been studied *in vitro*.

The smooth muscle of the urinary bladder contains both excitatory (α) and inhibitory (β) adrenergic receptors. The response elicited by adrenergic drugs which act on both types of receptors, e.g. adrenaline, depends firstly on the magnitude of the dose given. The inhibitory receptors are most sensitive and are activated by smaller doses than the excitatory receptors. Secondly the response obtained depends on which of the two types of receptors is dominant in the tissue. In the detrusor muscle it is the inhibitory response which dominates, whereas in the muscles from the bladder base it is the excitatory which is dominant. Thus with concentrations of adrenaline which activate the inhibitory as well as the excitatory response there is a relaxation in the detrusor muscle whereas in strips from the bladder base a contraction is obtained.

Blocking of one type of the receptors usually increases the sensitivity of the response elicited by the other type of receptors indicating that the response obtained in the untreated preparation is the resultant effect of the two opposing factors.

The findings have been discussed in relation to the embryological and anatomical differences of the bladder base and dome and particularly to the innervation of the smooth muscle and to the variation in physiological properties and functions of these cells.

Simultaneous stimulation with cholinergic drugs, which are excitatory in both types of muscles, increases the sensitivity of the inhibitory response but renders the urinary bladder less sensitive to the excitatory effect of adrenaline. These results have been correlated with the presumed changes in the membrane potential induced by the drugs.

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The Interaction between Different Metabolic Pathways of Catecholamines In the Brain Studied by Means of ^3H DOPA

By

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The fate of the dopamine formed from DOPA is determined by several processes 1) Some of the dopamine is taken up and stored for some time in the intraneuronal storage granules. 2) Part of this is converted to noradrenaline, which in turn is stored or metabolized according to process 1), 3) or 4) 3) Part of the dopamine is metabolized by oxidative deamination through monamine oxidase (MAO) or 4) by 3-O-methylation through catechol-O-methyl transferase (COMT) In the present study the role of these processes in the brain and the possible interaction between them has been investigated using selective inhibitors of each process Tritium-labelled L DOPA was used as a tool in continuation of a previous study (PERSSON & WALDECK 1968).

Material and Methods

All experiments were carried out on white mice with average weight of about 20 g. When not otherwise stated the ambient temperature was kept at $+22-24^\circ\text{C}$. The animals were divided into experimental groups, each of six mice. The catecholamine precursor ^3H DOPA was given intravenously dose of 5 $\mu\text{g}/\text{kg}$ either preceded or followed by the drugs to be tested. At certain time intervals the animals were killed by decapitation and the levels of ^3H -noradrenaline (^3H NA), ^3H -dopamine (^3H DA), ^3H -normetanephrine (^3H NM) and ^3H -methoxytyramine (^3H MT) in brain and heart determined as described elsewhere (PERSSON & WALDECK 1968).

The following drugs were used 1-(3,4-dihydroxyphenyl)-alanine (ring-2,5,6- ^3H) with specific activity of about 30 Ci/mmol (The Radiochemical Centre, Amersham), reserpine (F. edish CIBA Ltd.) isalamide (Swedish Pfizer Ltd.), α -propyldopacetamide (H 22/54, Hissle Ltd.), and bis(4-methyl-1-piperazinylthiocarbonyl)disulphide (EWP#15 Astra Ltd.)

) These drugs were generously supplied by the companies mentioned.

Results

Accumulation of ^3H -catecholamines after injection of ^3H DOPA in mice pretreated with reserpine and nialamide

Mice were given 10 mg/kg of reserpine i.p. alone or followed 4 hrs later by nialamide i.p. in doses from 10 to 200 mg/kg. Six hours after the injection of reserpine 5 $\mu\text{g}/\text{kg}$ ^3H DOPA was given i.v. One hour after this injection the animals were killed and ^3H NA, ^3H DA, ^3H NM and ^3H MT in the brain and heart determined. Some animals (controls) received only ^3H DOPA one hour before sacrifice.

Reserpine markedly reduced the amount of ^3H NA and ^3H DA recovered in the brain and of ^3H NA in the heart (fig. 1, 2 and 3). When the animals also received nialamide in increasing doses the amine levels rose, and in the brain they were maximal at a dose of 100 mg/kg of nialamide. In the heart an optimal ^3H NA level was already reached with a dose of 10 mg/kg of nialamide. With 200 mg/kg of nialamide the ^3H NA level in this tissue was significantly below the optimum ($P < 0.01$).

The optimal yield of ^3H NA in the brain was about 50 per cent of the control level though only 10 per cent of the value obtained after pre-

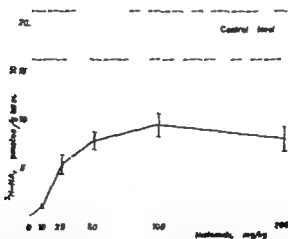


Fig. 1 Effect of reserpine and nialamide on the accumulation of ^3H -noradrenaline formed from ^3H DOPA in the mouse brain. Mice were given reserpine 10 mg/kg i.p. 6 hrs and nialamide in various doses i.p. 2 hrs before the i.v. administration of 5 $\mu\text{g}/\text{kg}$ ^3H DOPA. Controls received only ^3H DOPA. Sixty minutes after the injection of the labelled DOPA the animals were killed and the content of labelled amines in the brain and heart determined. The mean \pm S.E.M. of 3-9 experimental groups are shown (c.f. table 1) each group consisting of 6 animals.

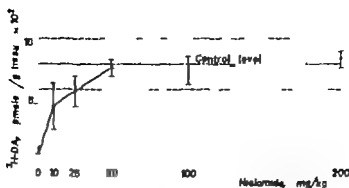


Fig. 2 Effect of reserpine and nialamide on the accumulation of ^3H -dopamine formed from ^3H DOPA in the mouse brain. For details see legend to fig. 1

treatment with nialamide alone (compare fig. 1 and 5) ^3H -dopamine reached the control level and about the fifth of the level found in nialamide-pretreated animals (compare fig. 2 and 5). In the heart the optimal yield of ^3H NA was relatively lower i.e. only some 10 per cent of the control value or about 5 per cent of the value obtained after nialamide alone (fig. 3 and 5). Because of a large scatter the ^3H DA levels in the heart are not shown.

When the MAO was intact the amounts of ^3H MT and ^3H NM found in the brain after injection of ^3H DOPA were relatively low irrespectively of whether reserpine had been given or not (table 1). Blockade of the

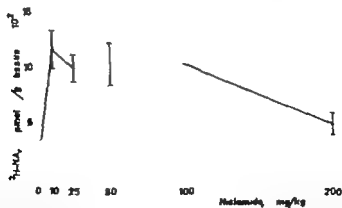


Fig. 3 Effect of reserpine and nialamide on the accumulation of ^3H -noradrenaline formed from ^3H DOPA in the mouse heart. 1 non-pretreated animals (there were 1.18 pmoles/g ^3H -noradrenaline). For details see legend to fig. 1

) 1 pmoles (picomoles) = 10^{-12} moles.

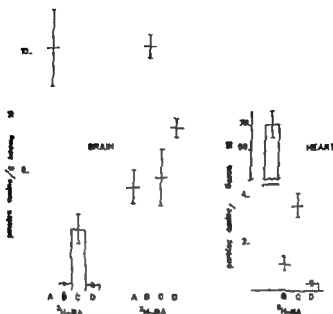


Fig. 4. Effect of reserpine + nialamide and a specific inhibitor of dopamine β -hydroxylase (EWP815) on the synthesis and accumulation of ^3H -noradrenaline (^3H NA) and ^3H -dopamine (^3H DA) in the brain and heart of the mouse. All animals received $5 \mu\text{g/kg}$ ^3H DOPA i.v. and were killed 60 min. later. Pretreatment was as follows. A. None, B. EWP815 50 mg/kg i.p. 30 min. previously, C. reserpine 10 mg/kg i.p. 6 hrs and nialamide 50 mg/kg i.p. 2 hrs before ^3H DOPA, and D. a combination of reserpine, nialamide and EWP815 with the same dose and time intervals as in B and C. The values are the mean \pm S.E.M. of 3 experimental groups, each group consisting of 6 animals.

MAO by nialamide in reserpine-treated animals resulted in an increase of both these amines. ^3H MT however rose much more than ^3H NM and reached a ten times higher level. The dose-response to nialamide appeared to be the same as that for their parent amines. In animals pretreated with nialamide alone (50 mg/kg) there was twice as much ^3H MT as in the corresponding reserpine-nialamide group (compare table 1 and 4). In contrast the levels of ^3H NM in the two groups were the same.

Effect of the dopamine β -hydroxylase inhibitor EWP815 on the accumulation of ^3H -catecholamines in non-pretreated and reserpine-nialamide pretreated mice after injection of ^3H DOPA

Mice received $5 \mu\text{g/kg}$ ^3H DOPA i.v. Some of the animals were pretreated with 50 mg/kg EWP815 i.p. 30 min. previously. Others received reserpine 10 mg/kg i.p. 6 hrs and nialamide 50 mg/kg i.p. 2 hrs previously.

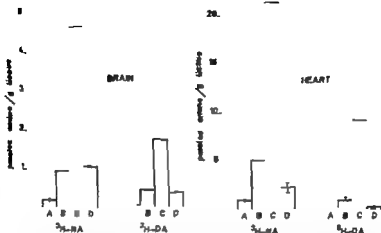


Fig. 5 Effect of α -propylidopacetamide and nialamide on the synthesis and accumulation of ^3H -noradrenaline (^3H -NA) and ^3H -dopamine (^3H -DA) in the brain and heart of the mouse. All animals received 5 $\mu\text{g/kg}$ ^3H -DOPA L and were killed 60 min. later. Pretreatment was as follows: A. None, B. α -propylidopacetamide 500 mg/kg i.p. 30 min., D. nialamide 50 mg/kg i.p. 2 hrs before the administration of ^3H -DOPA and C. combination of α -propylidopacetamide and nialamide with the same dose and time intervals as in B and D. Each dot represents one experimental group consisting of 6 animals (D is the mean \pm S.E.M. of 3 experimental groups).

In another group the animals received all the three drugs together at the same time intervals and dose schedules as described above. Sixty minutes after the injection of ^3H -DOPA the animals were killed and the ^3H -amine levels in the brains and hearts determined.

In the brain EWP815 reduced the amount of ^3H -NA recovered to about 3 per cent of that of non-pretreated animals (fig. 4) whereas ^3H -DA was increased by a factor of two. The results obtained with reserpine and nialamide were similar to those presented in fig. 1 though with a lower yield of ^3H -amines, which may be explained by the long interval between the two experimental series. EWP815 in combination with reserpine and nialamide reduced the yield of ^3H -NA to the same low level as that observed when this drug was given alone. There was a discernible though not statistically significant increase in ^3H -DA as compared with the group pretreated with reserpine and nialamide only.

In the heart ^3H -NA showed the same changes as in the brain except that the yield of the amine in the reserpine-nialamide pretreated animals was relatively lower (c.f. fig. 1 and 3).

Table 1

Effect of reserpine and nialamide on the accumulation of ^3H -normetanephrine and ^3H -methoxytyramine formed from ^3H -DOPA in the mouse brain. Mice were given reserpine in a dose of 10 mg/kg i.p. 6 hrs and nialamide in various doses i.p. 2 hrs before the administration of 5 $\mu\text{g/kg}$ ^3H -DOPA. Controls received only ^3H -DOPA. Sixty minutes after the injection of the labelled DOPA the animals were killed and the content of labelled amines determined in the brain and heart. The means \pm S.E.M. of the experimental groups are shown, each group consisted of 6 animals.

Dose of nialamide	^3H -normetanephrine pmoles/g	^3H -methoxytyramine pmoles/g	Number of experimental groups
Controls	0.05 ± 0.01	0.02 ± 0.00	9
0	0.10 ± 0.06	0.01 ± 0.00	7
10	0.04 ± 0.01	1.19 ± 0.18	6
25	0.18 ± 0.03	2.78 ± 0.7	5
50	0.24 ± 0.01	3.02 ± 0.18	9
100	0.34 ± 0.07	3.26 ± 0.27	6
200	0.27 ± 0.03	3.02 ± 0.19	5

1 pmoles (picomoles) = 10^{-12} moles.

The brain content of ^3H -NM in animals treated with reserpine-nialamide was reduced by EWP815 (table 2). ^3H -MT on the other hand appeared to increase following the administration of this drug.

The influence of the ambient temperature on the accumulation of ^3H -catecholamines in non-pretreated and reserpine-nialamide pretreated mice after injection of ^3H -DOPA

Mice were treated with reserpine, nialamide and ^3H -DOPA as described in the previous section except that the environmental temperature was kept at $+30^\circ$. The results are shown in table 3. When compared with the corresponding data obtained at an ambient temperature of $22-24^\circ$ (fig. 1, 2, 3 and 4, table 1 and 2) no definite differences could be detected.

Effect of H22/54 and nialamide on the accumulation of ^3H -catecholamines after administration of ^3H -DOPA

The inhibitor of COMT, H22/54, was given in a dose of 500 mg/kg i.p. 30 min. before ^3H -DOPA. In some experiments nialamide also in a dose of 50 mg/kg was given i.p. 2 hrs before ^3H -DOPA. Some animals received

Table 2

Effect of reserpine + nialamide and specific inhibitor of the dopamine β -hydroxylase (EWP815) on the synthesis and accumulation of ^3H -normetanephrine and ^3H -methoxytyramine in the brain of the mouse. All animals received $5 \mu\text{g/kg}$ ^3H DOPA i.v. and were killed 60 min. later. Pretreatment was as follows: None in the controls. EWP815 50 mg/kg i.p. 30 min. before ^3H DOPA in the second group. Reserpine 10 mg/kg i.p. 6 hrs and nialamide 50 mg/kg i.p. 2 hrs before ^3H DOPA in the third group, and a combination of reserpine, nialamide and EWP 815 with the same dose and time intervals as in the two preceding groups, in the fourth group. The mean values \pm S.E.M. of 3 experimental groups are shown, each group consisting of 6 animals.

Pretreatment	H-normetanephrine pmoles/g	^3H -methoxytyramine pmoles/g
None	0.01 ± 0.00	0.01 ± 0.00
EWP 815	0.00	0.01 ± 0.00
Reserpine + nialamide	0.06 ± 0.01	1.60 ± 0.36
Reserpine + nialamide + EWP 815	0.01 ± 0.00	2.61 ± 0.07

 $p < 0.05$ $p < 0.05$

only ^3H DOPA and others nialamide and ^3H DOPA (fig. 5 and table 4) Sixty minutes after the injection of the labelled precursor the animals were killed and the ^3H amine levels in the heart and brain determined.

In the brain both H22/54 and nialamide when given separately increased the ^3H NA about five-fold as compared with non-pretreated animals. The two drugs in combination raised the ^3H NA level almost 25 times i.e. the

Table 3

The influence of the ambient temperature on the accumulation of H-catecholamines in the brain and heart of non-pretreated and reserpine-nialamide-pretreated mice after injection of ^3H DOPA. Controls received only $5 \mu\text{g/kg}$ ^3H DOPA i.v. In the other group reserpine 10 mg/kg i.p. 6 hrs and nialamide 50 mg/kg i.p. 2 hrs before ^3H DOPA was given. The ambient temperature was $+30^\circ\text{C}$. The mean values \pm S.E.M. of 3 experimental groups are shown, each consisting of 6 animals.

Pretreatment	Brain				Heart
	H-nor adrenaline	H-dop- amine	H-nor- metanephrine	H-metho- xytyramine	H-nor adrenaline
None	0.25 0.02	0.11 0.01	0.05 0.01	0.0 0.00	1.31 0.18
Reserpine nialamide	0.10 0.00	0.10 0.00	0.16 ± 0.00	2.76 ± 0.06	0.09 ± 0.01

Table 4

Effect of α -propylidopacetamide (H 22/54) and nialamide on the synthesis and accumulation of ^3H -normetanephrine and ^3H -methoxytyramine in the brain of the mouse. All animals received 5 $\mu\text{g}/\text{kg}$ ^3H -DOPA i.p. and were killed 60 min. later. Pretreatment was as follows: None in the controls. In the second group H 22/54 500 mg/kg i.p. 30 min. before ^3H -DOPA. In the third group nialamide 50 mg/kg i.p. 2 hrs before ^3H -DOPA. In the fourth group a combination of H 22/54 and nialamide in doses and at time intervals as above. The means \pm S.E.M. are shown.

Pretreatment	H-normetanephrine	H-methoxytyramine	Number of experimental groups
None	0.05 ± 0.00	0.02 ± 0.00	2
H 22/54	0.01 ± 0.00	0.00	2
Nialamide	0.27 ± 0.05	6.28 ± 0.18	3
Nialamide + H 22/54	0.05 ± 0.01	0.14 ± 0.01	2

Table 5

The ratio $(^3\text{H-NA} + ^3\text{H-NM})/^3\text{H-DA}$, calculated from the determinations shown in the preceding figures and tables.

Pretreatment	The experimental data are shown in		$\text{H-NA} + \text{H-NM}$ H-DA
	figure	table	
None	1	1	3.7
Reserpine	1	1	1.4
Reserpine + nialamide 10 mg/kg.	1	1	1.0
Reserpine + nialamide 25 mg/kg.	1	1	4.0
Reserpine + nialamide 50 mg/kg.	1	1	4.0
Reserpine + nialamide 100 mg/kg.	1	1	5.5
Reserpine + nialamide 200 mg/kg.	1	1	3.9
Nialamide	3	4	3.4
Reserpine + nialamide 50 mg/kg at + 30	—	3	2.6
EWP 815	4	2	0.06
Reserpine + nialamide 50 mg/kg + EWP 815	4		0.2
H 22/54	3	4	2.0
H 22/54 + nialamide	3	4	2.6

product of the separate effects. ^3H DA in the brain showed changes similar to those of ^3H NA. In the heart too both ^3H NA and ^3H DA were considerably increased after the inhibition of MAO and COMT and much more than after inhibition of either enzyme alone.

After nialamide there was a tremendous increase (more than 300 times) of ^3H MT in the brain as compared with the non-pretreated group. The increase in ^3H NM was much less marked. Additional treatment with H22/54 brought the 3-O-methyl derivatives back to low levels (table 4).

The ratio (^3H NA + ^3H NM)/ ^3H DA

Table 5 shows the ratio (^3H NA + ^3H NM)/ ^3H DA obtained from the experiments reported in the previous chapters. This ratio is assumed to be a measure of the activity of the DA β -hydroxylase (see discussion). In most of the experiments the ratio was about 3. When reserpine was given alone or together with a small dose of nialamide before ^3H DOPA, the ratio (^3H NA + ^3H NM)/ ^3H DA decreased to about 1. Pretreatment with EWP815 however reduced the ratio 10–50 times.

Discussion

The present study illustrates the well known decisive importance of the storage mechanism of the amine granules for the fate of catecholamines in tissues. When this mechanism was blocked with reserpine, the recovery of ^3H DA and ^3H NA was considerably reduced. Evidently the loss of the amines was largely brought about by MAO since inhibition of this enzyme by nialamide considerably increased the recovery of both amines. In previous investigations on peripheral tissues it has been shown that the effect of MAO inhibition is not purely protective (CARLSSON & WALDECK 1967; CARLSSON, LINDQVIST & WALDECK 1968). Accumulation of endogenous amines may lead to displacement of the labelled amines, with a resultant decrease in recovery following larger doses of a monoamine oxidase inhibitor. This phenomenon was also clearly seen in the present study in as far as the heart is concerned. In the brain where the uptake of amines from the blood is limited by the blood brain barrier at most a tendency in this direction could be seen following the highest dose of nialamide used i.e. 200 mg/kg.

By means of selective inhibitors of MAO (nialamide) and COMT (H22/54) both oxidative deamination and 3-O-methylation were shown to be of importance in the metabolism of catecholamines in the brain during the fairly short interval studied. That MAO and COMT do indeed play a

Table 4

Effect of α -propylidopacetamide (H 22/54) and nialamide on the synthesis and accumulation of H-normetanephrine and ^3H -methoxytyramine in the brain of the mouse. All animals received 5 $\mu\text{g/kg}$ H DOPA I.v. and were killed 60 min. later. Pretreatment was as follows: None in the controls. In the second group H 22/54 500 mg/kg i.p. 30 min. before H DOPA. In the third group nialamide 50 mg/kg i.p. 2 hrs before H DOPA. In the fourth group a combination of H 22/54 and nialamide in doses and at time intervals as above. The means \pm S.E.M. are shown.

Pretreatment	H-normetanephrine	^3H -methoxytyramine	Number of experimental groups
None	0.04 \pm 0.00	0.02 \pm 0.00	2
H 22/54	0.01 \pm 0.00	0.00	2
Nialamide	0.27 \pm 0.05	6.28 \pm 0.18	3
Nialamide + H 22/54	0.05 \pm 0.01	0.14 \pm 0.01	

Table 5

The ratio $(^3\text{H NA} + ^3\text{H NM})/^3\text{H DA}$, calculated from the determinations shown in the preceding figures and tables.

Pretreatment	The experimental data are shown in		$^3\text{H NA} + ^3\text{H NM}$ H-DA
	figure	table	
None	1	1	3.7
Reserpine	1	1	1.4
Reserpine + nialamide 10 mg/kg	1	1	1.0
Reserpine + nialamide 25 mg/kg	1	1	4.0
Reserpine + nialamide 50 mg/kg.	1	1	4.0
Reserpine + nialamide 100 mg/kg	1	1	3.5
Reserpine + nialamide 200 mg/kg	1	1	3.9
Nialamide	5	4	3.4
Reserpine + nialamide 50 mg/kg at + 30	—	3	2.6
EW 815	4	2	0.06
Reserpine + nialamide 50 mg/kg + EW 815	4		0.2
H 22/54	5	4	2.0
H 22/54 + nialamide	5	4	2.6

function, destruction by MAO will dominate the metabolism of DA and thus reduce the total yield of NA without necessarily influencing the yield relative to the intraneuronal DA concentration

Summary

^3H DOPA of high specific activity was given i.v. in small doses ($5\text{ }\mu\text{g/kg}$) to mice. One hour later the animals were killed and ^3H noradrenaline (^3H NA) ^3H -dopamine (^3H DA) ^3H -normetanephrine (^3H NM) and ^3H -methoxytyramine (^3H MT) determined in the brain and heart.

Pretreatment with reserpine largely reduced the accumulation of ^3H DA and ^3H NA. After additional treatment with the inhibitor of monoamine oxidase (MAO), nialamide ^3H NA and ^3H DA again accumulated. Inhibition of DA β -hydroxylase with bis(4-methyl-1-piperazinylthiocarbonyl)-disulphide (EWP815) markedly reduced the yield of ^3H NA with a concomitant increase in ^3H DA. When the efficiency of the DA β -hydroxylase was expressed as the ratio $(^3\text{H}$ NA + ^3H NM)/ ^3H DA EWP815 but not reserpine caused a clearcut inhibition. It is suggested that the interaction of reserpine with the synthesis of NA is more complex than a simple blockade of the uptake of DA into the amine-storing granules, i.e. the site of DA β -hydroxylase.

In addition, animals were pretreated with nialamide and the inhibitor of catechol-O-methyl transferase (COMT) α -propyldopacetamide (H22/54). Inhibition of either enzyme alone caused a fivefold increase of ^3H DA and ^3H NA recovered. After nialamide there was also a remarkably high level of ^3H MT. Simultaneous blockade of the two enzymes increased the yield of ^3H NA and ^3H DA by about 25 times in both the brain and heart as compared with animals which received ^3H DOPA only. The interaction of MAO and COMT in the NA and DA metabolism is discussed.

Acknowledgements

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High Frequency Electro-shock Seizures and their Antagonism during Postnatal Development in the Mouse.

I. Parameters for Electro-shock and Effect of Diphenylhydantoin

By

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Studies of ontogenic changes have greatly contributed to knowledge of the structure and function of the central nervous system (see HIRVICH & HIRVICH 1964). Very few studies on the effects of drugs on the central nervous system during postnatal development have however been undertaken, probably because of methodological difficulties. Since seizures are not uncommon in childhood, experimental studies on seizure susceptibility and antagonism during maturation have a practical as well as an intrinsic interest. Studies of this nature have been reported but the early neonatal period has usually been omitted (MILLICHAPE 1957 VERNADAKIS & WOODBURY 1961 1963 1964 & 1965). The methodological difficulty of eliciting seizures in newborn animals can be overcome by spinal cord stimulation (VERNAKAKIS 1962) or by the use of chemical convulsants (FERNGREN 1965 & 1968) but the results are not easily interpreted.

For this reason we have modified the electro-shock procedure introduced by PAALZOW (1966) in order to see if it was possible to induce seizures in intact mice, even in the newborn period, by choosing suitable electrical parameters. We also wanted to see if such electro-shock seizures could be modified by anticonvulsants. In the following electrical parameters for the production of tonic forelimb seizures during postnatal development and a dose-dependent reduction of the duration of such seizures with diphenylhydantoin are described. Developmental studies of the effect and time course of some well-known anticonvulsants with this method will be reported in an accompanying paper

Materials and Methods

Apparatus.

For electrical stimulation of the animals a Kistner Type 421 pulse stimulator was used which gives a series of square wave pulses which can be modified through a range of parameters. An oscilloscope (Solartron CD 1400) was used to check the adjustment of parameters. Platin m needle electrodes with a diameter of 0.4 mm and a length of 12 mm (Grass II 2B) were used. They were applied subcutaneously in the temporal region, with the positive electrode placed medially at the edge of the left ear and with the negative electrode placed at the lateral edge of the right ear.

Animals.

Mice of the NMRI strain were used throughout the study. On the day after birth they were received together with their mothers from a local breeder and then kept with their mothers until the experiments were performed. Groups of mice 1, 3, 5, 9, 12, 15, 18 and 21 days old were studied. No attempt was made to separate the sexes. The experiments were performed at a temperature of 20–25°C.

Determination of electro-shock parameters.

Investigations were carried out on 1,200 mice of different ages. Frequency, pulse width, and duration of shock of the square wave stimulus were studied. The fixed values for these parameters were investigated in randomized order. Between every stimulation there was a pause of 45 minutes. The percentage of animals with forelimb and hindlimb extension and with urination were registered. When one parameter was studied, the others were fixed at definite values as described in figures 1–3. The voltage was selected so that when the parameters were optimal almost 100% of the animals had seizures.

Anticonvulsant effects

The reduction in duration of the extension of forelimbs was chosen as a measure of the anticonvulsant effect. Threshold voltage for seizures was determined individually by stepwise increases of voltage with electrical parameters according to table 1. Only in 18 and 21-day-old mice was the threshold voltage increased by 20% to ensure convulsions with repeated stimulation. Diphenylhydantoin was given subcutaneously in doses of 6.5 mg/kg to each of 12–15 animals. The same number of animals were given the solvent as controls. The electro-shock was repeated 0.5, 1, 1.5, 2, 3, 4, 5 and 6 hours after the administration of the drug and the duration of the tonic extension measured with stop-watch. Up to 5 hours after administration the young animals were kept separated from their mothers but after the shock at 5 hours they were returned to their mothers.

Dose-response curves.

The reduction in per cent in the duration of tonic forelimb extension from the pre-test value was taken as an index of response, at three dose levels 3.25, 6.5 and 13 mg/kg. The duration of seizures was measured 30, 60 and 90 minutes after injection of the drug and the result obtained at 60 minutes was used for the calculation of regression lines according to FINNEY (1947). For each age about 30 young mice were used.

Test solution.

Diphenylhydantoin = phenytoinum NPN = 5,5-diphenyl-hydantoin. A solution of the sodium salt from the commercial preparation spanutin ® (Parke-Davis) was dissolved

is the solvent prepared according to the manufacturer. As solvent 40% (w/v) propylene glycol and 10% ethyl alcohol (w/v) in water was used. For 1 and 3-day-old mice a 0.1% (w/v) solution, for 5-day-old mice 0.5% solution and from the age of 9 days a 1% solution was used. The volume injected per animal varied between 2.5 and 10 microlitres.

Results

1 Parameters of electro-shock.

A Frequency

One 3 and 5-day-old mice had a maximum incidence of forelimb seizures at a frequency of 100 p/sec. (fig. 1 A). At higher frequencies the susceptibility to seizures diminished and at 800 p/sec. 1-day-old animals had no seizures at all. In older mice forelimb seizures occurred at higher frequencies (fig. 1 B and C) and the stimulus frequency for maximum seizure susceptibility seems to increase with maturation.

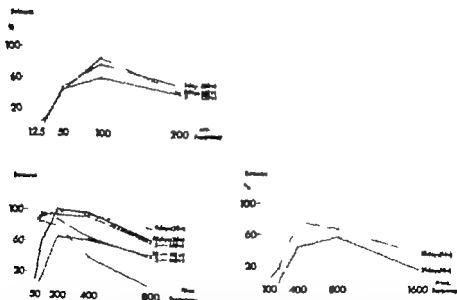


Fig. 1 The influence of the frequency of electro-shock on the development of seizures in 1 to 21-day-old mice. A, Pulse width 2 msec. and duration of the stimulus 800 msec. Each point represents the percentage of seizures in 40 animals; B, Pulse width 0.5 msec. and duration of the stimulus 800 msec. Each point represents the percentage of seizures in 48 animals; C, Pulse width 0.5 msec. and duration of the stimulus was 800 msec. for 18- and 400 msec. for 21-day-old mice. Each point represents the percentage of seizures in 35 animals.

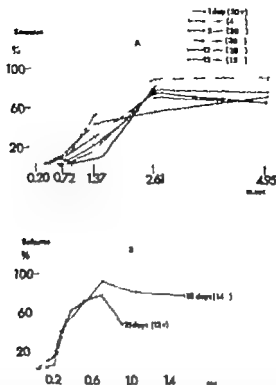


Fig. 2. The influence of pulse width of the single pulses of the electro-shock on the development of seizures in 1 to 21-day-old mice. *A*, Frequency was 100 p/sec. and duration of the electro-shock 800 msec. Each point represents the percentage of seizures in 36 animals; *B*, Frequency was 400 p/sec. for 18 and 800 p/sec. for 21-day-old mice and duration of the electro-shock 800 msec. Each point represents the percentage of seizures in 27 animals.

B Pulse width

Six logarithmically spaced values for the pulse width from 0.20 to 4.95 msec. were tested. Up to the age of 15 days the percentage of animals in convulsions increased as the pulse width was increased (fig. 2 A). The maximum number of convulsions in 18-day-old animals was seen at a pulse width of 0.7–1.5 msec. With 21-day-old mice most seizures occurred at 0.5–0.7 msec. pulse width (fig. 2 B). The pulse width cannot be set at any arbitrary value but is limited by the pulse interval i.e. the distance between two pulses. It cannot cover more than 90% of this interval, but because of technical factors it should not exceed 50%.

C. Duration of shock

Five different durations of the total electrical stimulus were investigated. The percentage of mice with convulsions increased at durations longer

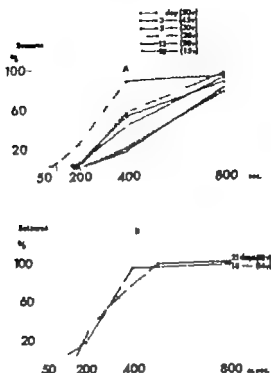


Fig. 3. The influence of the duration of electro-shock on the development of seizures in 1 to 21-day-old mice. A, Frequency was 100 p/sec. and pulse width 2 msec. Each point represents the percentage of seizures in 40 animals; B, Frequency was 400 and 800 p/sec. respectively and pulse width 0.8 msec. for 18-day-old mice and 0.6 msec. for 21-day-old mice. Each point represents the percentage of seizures in 36 animals.

than 200 msec. in 15-day-old and younger mice, (fig. 3 A). 18 and 21-day-old mice had optimum seizure susceptibility with durations between 400 and 800 msec. (fig. 3 B).

From the results of these investigations, suitable electro-shock parameters for developing mice were selected and are given in table 1. For 12 and 15-day old animals the chosen frequency is not optimum as seen from fig. 1 B but was selected for comparative reasons. Generally the values which gave maximum seizure susceptibility were chosen for the different parameters and for 21-day-old animals they are the same as those used for adult mice.

2. Seizure patterns.

With this method it was possible to elicit tonic forelimb seizures in intact mice even in the newborn period. After applying the shock stimulus,

Table 1

Definite parameters for high frequency electro-shock seizures in developing mice.

Age	Frequency (p/sec.)	Pulse width (msec.)	Duration of shock (msec.)
1 3 5 9 12, 15	100	4	800
18	400	0.8	400
21	800	0.6	400

the forelimbs were observed to be extended tonically and caudally along the body and when the seizure ended, the forelimbs relaxed and moved forwards. In 15-day-old and older mice the relaxation was accompanied by clonic movements. From the age of 9 days hindlimb seizures (tonic extension) occurred in a few animals and later these increased in frequency during development. The relation between the percentage of hindlimb seizure and that of forelimb seizure is shown in fig. 4 this represents the maximum points in the investigations of electrical parameters.

From the age of 18 days a seizure pattern corresponding to that of adult animals was manifest with the exception that in a few animals, the forelimb seizure was not accompanied by tonic hindlimb extension. In 21-day-old mice a forelimb seizure was always accompanied by a hindlimb seizure. Urination occurred at all ages during the seizure and also

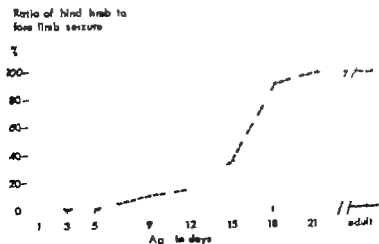


Fig. 4. Percentage of animals with forelimb seizures which also had hindlimb seizures.

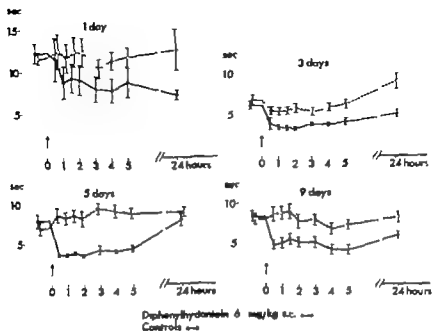


Fig. 5. Mean duration of tonic forelimb seizures in 1 to 9-day-old mice after diphenylhydantoin 6.5 mg/kg subcutaneously. Controls were injected with the solvent. Vertical bars indicate S.E.M.

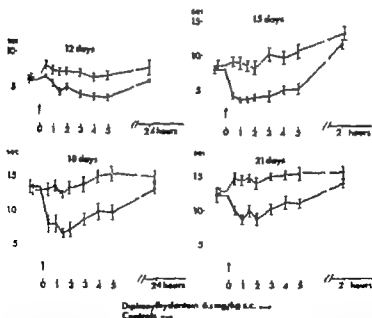


Fig. 6. Mean duration of tonic forelimb seizures in 12 to 21-day-old mice after diphenylhydantoin 6.5 mg/kg subcutaneously. Controls were injected with the solvent. Vertical bars indicate S.E.M.

Table 2

Comparison of slopes (b) for dose response lines for the reduction of seizure duration by diphenylhydantoin 60 minutes after subcutaneous injection of mice at different ages. Diphenylhydantoin was given in the following doses 3.25 6.5 and 13 mg/kg.

Age in days	n	b \pm s.e.	Comparison of age groups (Student's t-test)	P
1	30	- 37.54 \pm 7.62	1-3	0.2-0.1
3	30	- 35.48 \pm 8.12	3-5	<0.001
5	30	-129.40 \pm 19.49	5-9	0.01-0.001
9	30	- 59.30 \pm 15.24	9-12	0.4-0.3
12	30	- 78.87 \pm 15.59	12-15	0.2-0.1
15	30	- 52.16 \pm 8.73	15-18	<0.001
18	30	-124.25 \pm 11.56	18-21	0.7-0.6
21	27	-135.47 \pm 25.98		-

with electrical parameters that were not optimal for seizures. Arrest of respiration was seen in conjunction with the seizure

3 Dose response curves

In all age groups, linear regressions were found for the log-dose response relationship for the effect of diphenylhydantoin. The slopes of the calculated lines for diphenylhydantoin 60 minutes after its administration are given in table 2 together with a statistical comparison between slopes of neighbouring ages. The regression line for 5-day-old mice has a slope that differs significantly from the slopes for 3 and 9-day-old animals. The slope for 18-day-old mice also differs significantly ($P < 0.001$) from that of 15-day-old mice.

4 Effect of diphenylhydantoin.

The effect of diphenylhydantoin in a dose of 6.5 mg/kg given subcutaneously on the duration of electro-shock as compared with the effect of the control solution is shown in fig. 5 for 1 to 9-day-old mice and in fig. 6 for 12 to 21-day-old mice. These figures show that diphenylhydantoin generally reduces the duration of the forelimb seizure to about half of the control value, although there is a smaller effect in 1 and 21-day-old animals. In most age groups the effect is still evident 24 hours after injection.

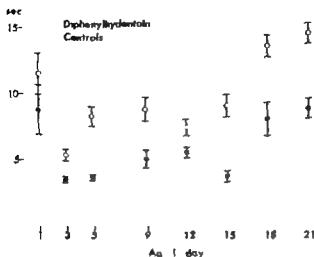


Fig. 7 Mean duration of forelimb seizures 60 minutes after diphenylhydantoin 6.5 mg/kg subcutaneously compared with solvent-injected control mice during the postnatal period. Vertical bars indicate S.E.M.

In no case was it possible to abolish completely the tonic forelimb extension induced by the electro-shock. The effects one hour after administration in all investigated age-groups have been plotted in fig. 7 to illustrate the anticonvulsant effect of the same dose of diphenylhydantoin during maturation.

Discussion

Electro-shock parameters

It has not been possible with the previous methods used for the induction of electro-shock seizures, to elicit convulsions in intact mice and rats during the newborn period. With the electro-shock procedure introduced by WOODBURY & DAVENPORT (1952) which uses an alternating current of 60 cyc./sec., MILLICHAPE (1957) could not elicit tonic or symmetrical clonic seizures in rats under the age of 10 days. The procedure of EARLIN & FRESTON (1961) was modified by VERNADAKIS (1962) for developing rats. In decapitated rats it was possible to produce tonic flexion and extension of hindlimbs in 1-day-old rats with square wave stimuli, with frequencies from 25 to 300 p/sec.

Using square wave stimuli of low frequency (6 p/sec.) VERNADAKIS & WOODBURY (1965) could only induce seizures in developing mice the age of 13 days. It should be mentioned in this connection that

cells from human epileptogenic foci fire intermittent bursts of high frequency up to 800 p/sec. (WARD & SCHMIDT 1961)

During the early postnatal period the mice respond to lower frequencies (100 p/sec.) than later on, when frequencies of 400–800 p/sec. give optimum seizures. VERNADAKIS (1962) obtained similar results with spinal cord stimulation in rats. This finding could be due to physical phenomena since a current of low frequency penetrates deeper than a current of high frequency. The results can also be interpreted as indicating that immature brain cells and structures are more easily stimulated by stimuli of somewhat lower frequencies.

Regardless of age the pulse width should be 30–60 μ of the pulse interval in order to obtain a maximum effect (seizures) a result which is in agreement with the findings in adult mice (PAALZOW 1966)

The duration of the shock depends on the voltage used, which is a measure of the amount of current supplied. A total duration of the stimulus of 0.8 sec. is enough in all age groups, when the voltages given in fig. 3 are used, but in older animals (over 18-day-old) 0.4 sec. is also enough.

The voltage used to induce threshold seizures in our experiments varies from 50 v in newborn mice to 30–10 v in mice 5 to 21 days of age. In adult mice the threshold voltage with this method is about 10 v (PAALZOW 1966). That a higher voltage is needed in younger animals is in agreement with the findings of MEYERSON (1968) in fetal sheep in which he found threshold voltages of 40–70 v in the smaller fetuses for inter-hemispheric response as compared to 10 v in adult sheep. As MEYERSON points out there are several characteristics of the immature brain which could account for its low excitability such as special membrane properties, the small surface area and the primitive dendritic tree of the immature neurons, as well as the extensive extracellular space and the high water content.

Seizure pattern.

The seizure pattern which in newborn animals consists of forelimb extension and urination changes during development having adult characteristics from the age of 18 days. The maturation of the high frequency electro-shock seizure pattern fits in with other parameters expressing the ontogeny of brain function in the mouse. KOBAYASHI *et al* (1963) have shown that the mouse has an electro-corticogram of the adult type from 16–17 days of age and that the evolution of the brain is histologically complete from the age of 15–17 days. FOX (1965) studied reflex responses during the postnatal development of the mouse and found "marked

changes in reflex responses at 13-16 days of age when adultlike behaviour patterns appear"

Effect of diphenylhydantoin

The effect of diphenylhydantoin on conventional electro-shock seizures during postnatal development in mice and rats has been studied in several investigations by VERNADAKIS & WOODBURY (1964a, 1964b & 1965). In mice the drug was found to elevate a.c. thresholds for seizures from 13 to 18 days of age and also to elevate low frequency thresholds (according to TOMAN 1951) in mice older than 17 days (VERNADAKIS & WOODBURY 1965). Diphenylhydantoin reduces the duration of hindlimb extension elicited by spinal cord stimulation in 4-day-old and older rats (VERNADAKIS & WOODBURY 1964a). As pointed out by VERNADAKIS & WOODBURY (1964) the duration of flexion and/or extension is a measure of seizure intensity. Diphenylhydantoin in a dose one third of that used by previous investigators has in the present study been shown to reduce the intensity of high frequency electro-shock seizures in all age groups except in newborns. This also clearly demonstrates the higher sensitivity of this method for developing animals.

In the studies of dose-effect relations it was found that a higher dosage of diphenylhydantoin was needed in newborn animals to reduce the intensity of seizures. This finding is in contrast to the beneficial effect of phenobarbital against pentetrazol and picrotoxin induced convulsions in newborns (FERNGREN 1965 & 1968). No explanation for this can be offered at present.

Summary

A new electro-shock procedure utilizing high frequency square wave stimulation has been modified for mice during postnatal development. The importance of frequency, pulse width and duration of the stimulus for provoking seizures was studied. It was possible to elicit a tonic forelimb extension in mice at 1, 3, 5, 9, 12, 15, 18 and 21 days of age. Frequencies between 50 and 1600 p/sec. were investigated and younger animals were found to be more sensitive to lower frequencies. The seizure pattern changed with maturation and showed adult characteristics in 15-day-old animals. Diphenylhydantoin was shown to give a dose dependent reduction of the duration of the seizure in all age-groups. The effect lasted for at least 24 hours in the youngest mice to the age of 12 days and less in older mice. 6.5 mg/kg subcutaneously had a beneficial effect in all age-groups except in newborns.

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High Frequency Electro-shock Seizures and their Antagonism during Postnatal Development in the Mouse. II. Effects of Phenobarbital Sodium, Mephobarbital, Trimethadione, Dimethadione, Ethosuximide and Acetazolamide

By

Harry Ferngren and Lemert Paalzow

(Received January 13 1969)

In our previous paper (FERNGREN & PAALZOW 1969) a new electro-shock procedure for the induction of seizures in newborn and very young mice was described and a dose-dependent reduction of seizure duration by diphenylhydantoin (phenytoinum NFN) was demonstrated. In this paper results are reported of experiments in which this procedure was used for some anticonvulsants which have been studied previously in immature animals with other methods. Ethosuximide was also included since this drug is increasingly used for the treatment of petit mal during childhood.

Materials and Methods

The experiments were performed on NMRI mice. On the day after birth they were received from a local breeder together with their mothers and then kept with their mothers until the experiments were performed. The following age groups were studied: 1, 3, 5, 9, 12, 15 and 21-day-old mice. No attempt to separate the sexes was made. A total of about 1,200 young mice were used in this study.

The electro-shock procedure was the same as that reported in the previous paper (FERNGREN & PAALZOW 1969). The duration of seizure in drug-injected animals and solvent-injected controls were measured 0.5, 1, 1.5, 2, 3, 4, 5 and 24 hours after injection. The young animals were kept separated from their mothers up to five hours after the injection.

Test solutions.

1. *Phenobarbital* = phenobarbitalum NFN = (5-ethyl-5-phenylbarbituric acid). A 1% (w/v) solution of the sodium salt in 0.9% NaCl was used for all age groups except for the 1-day-old animals which received a 0.5% solution.

2. *Mephobarbital* = mephumentalum NFN = (5-ethyl-1-methyl-5-phenylbarbituric acid). 1 g of the drug was dissolved in 4.06 ml 1.0 N NaOH and 40 g propylene glycol were added during slow heating. The solution was made up to 100 ml with distilled water. This 1% solution was used for all age groups except for the 1-day-old animals which received a 0.5% solution.

3. *Trimethadione* = trimethadionum NFN = (3,5,5-trimethyl-2,4-oxazolidinedione)¹⁾. A 5% (w/v) solution in 0.9% NaCl.

4. *Dimethadione* = dimethadionum NFN = (5,5-dimethyl-2,4-oxazolidinedione)²⁾. A 5% (w/v) solution in 0.9% NaCl.

5. *Acetazolamide* = acetazolamidum NFN = (2-acetamido-5-sulfamoyl-1,3,4-thiadiazol). A 10% (w/v) solution of the sodium salt in 0.9% NaCl.

6. *Ethosuximide* = ethosuximidum NFN = (2-ethyl-2-methyl-succinimide). A 10% solution in 0.9% NaCl was used for all age groups, except for 1 and 3-day-old mice for which a 5% solution was used.

Results

The effect of phenobarbital (13 mg/kg) on high frequency seizures during development is demonstrated in fig. 1 and 2. The best effect is seen in 5, 15 and 21-day-old animals. The duration of seizure was reduced to a rather constant level for 5 hours. The duration of the effect is less than 24 hours from 9 days of age and at least 24 hours in 1 and 3-day-old mice.

The effect of mephobarbital, the methylated analogue of phenobarbital, during development is depicted in fig. 3 and 4. This drug also has its most marked effect in 5, 15 and 21-day-old animals. There is no unequivocal effect in 9-day-old animals. The duration of effect is less than 24 hours from 5 days of age but is longer in the youngest age groups.

Fig. 5 and 6 demonstrate the effect of trimethadione (400 mg/kg) on seizure duration during development. This dose gives a reduction in seizure duration in all age groups though least in 9-day-old animals. The duration of the effect is longer than 24 hours in 1, 3, 5 and 15-day-old animals.

The effect of dimethadione (400 mg/kg) is shown in fig. 7 and 8. This dose gives a reduction of seizure duration in all age groups. The duration of action is more than 24 hours except in 5-day-old animals.

The effect of ethosuximide (266 mg/kg) is depicted in fig. 9 and 10. Practically no effect was found in newborn mice or in 21-day-old mice. The effect becomes slowly evident and is maximal after 1.5-2 hours. The duration of effect is less than 24 hours in all age groups.

1) Supplied through the courtesy of Abbott Laboratories in Scandinavia.

2) Supplied through the courtesy of dr. Robert L. Alberti, Abbott Laboratories, North Chicago.

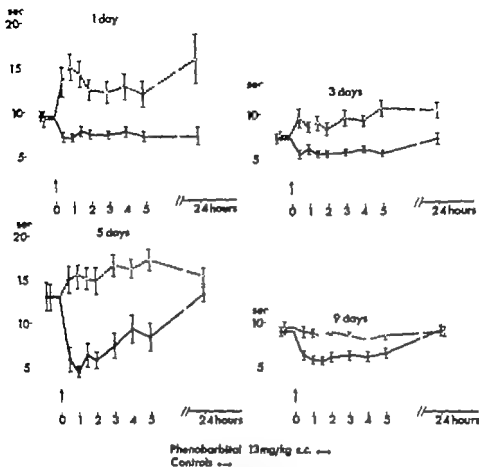


Fig. 1 Mean duration of tonic forelimb seizures in 1 to 9-day-old mice after phenobarbital 13 mg/kg subcutaneously. Controls were injected with physiological saline. Vertical bars indicate S.E.M.

Acetazolamide in the massive dose of 1 g/kg has the best effect of all the drugs investigated from the age of 15 days (fig. 11 and 12). The duration of seizure was reduced so much at 5 hours in 21-day-old mice that only a very short forelimb extension which could not be accurately measured was seen. From 5 days of age the duration of effect was less than 24 hours.

Control animals (litter-mates) which were handled exactly as the drug-injected animals except that they were injected with the solvent, were also tested for seizure susceptibility. An increase in the duration of forelimb extension after the pretest procedure was often observed in the

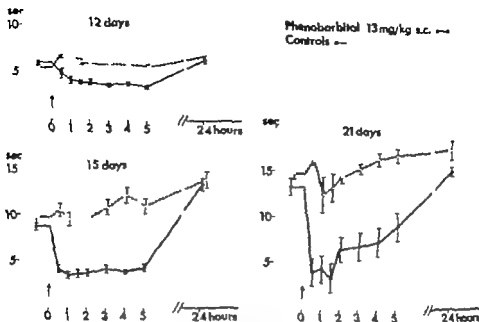


Fig. 2. Mean duration of tonic forelimb seizures in 12 to 21-day-old mice after phenobarbital 13 mg/kg subcutaneously. Controls were injected with physiological saline. Vertical bars indicate S.E.M.

control animals. The mean seizure durations of all the saline-injected controls of the same age but from different experiments were therefore calculated and are depicted in fig. 13. Each age group among the controls seems to have its own characteristic pattern of variation in seizure duration with repeated electro-shock. The pretest seizure durations for neighbouring ages have been compared statistically in table 1. All differences were found to be significant. Moreover pretest durations were shown not to differ for 3, 9 and 15-day-old animals. Therefore the values 30 minutes after injection for these age groups were also compared. A significant difference was found between 3-day-old and 15-day-old mice ($P < 0.05$) and between 9-day-old and 15-day-old mice ($P < 0.01$).

The most marked change in seizure durations was observed in 3-day-old and 5-day-old mice. The mean increase in duration of seizure from the pretest shock to the shock at 30 minutes was calculated and compared for these two age groups. No statistical difference was found. The finding that in 9-day-old mice there is no change in seizure duration with repeated electro-shock is also of considerable interest.

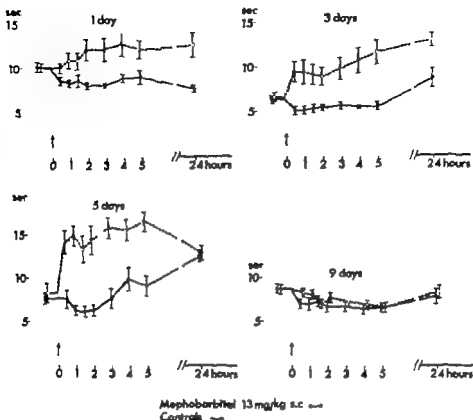


Fig. 3. Mean duration of tonic forelimb seizures in 1 to 9-day-old mice after mephobarbital 13 mg/kg subcutaneously. Controls were injected with the solvent. Vertical bars indicate S.E.M.

Discussion

The results indicate the existence of certain critical age periods — newborns, 9-day-old and 21-day-old mice — in which certain of the anti-convulsants have a poor effect although they are active at other ages. This may of course be a question of dosage. The single doses for the anti-convulsants were chosen with the background knowledge of oral ED₅₀s for the tonic hindlimb component of high frequency electroshock in adult animals (PAALZOW 1966) as well as the knowledge of the subcutaneous ED₅₀s for pentetrazol seizure in developing mice (FERN-OREN 1965 & 1968). The main reason for using a fixed dose throughout the postnatal period was that no rule of extrapolation exists for some

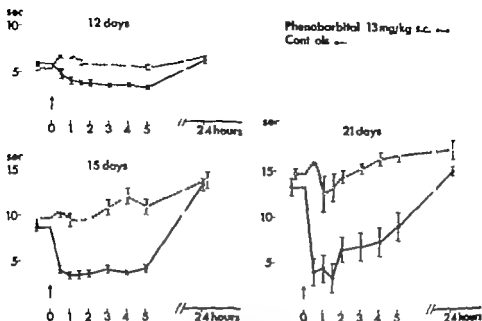


Fig. 2. Mean duration of tonic forelimb seizures in 12 to 21-day-old mice after phenobarbital 13 mg/kg subcutaneously. Controls were injected with physiological saline. Vertical bars indicate S.E.M.

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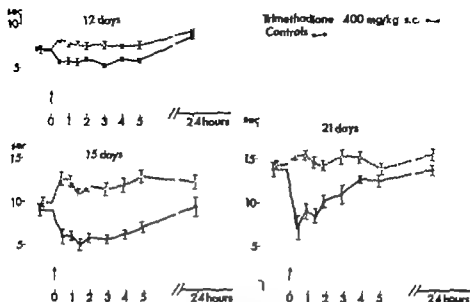


Fig. 6. Mean duration of tonic forelimb seizures in 12 to 21-day-old mice after trimethadione 400 mg/kg subcutaneously. Controls were injected with physiological saline. Vertical bars indicate S.E.M.

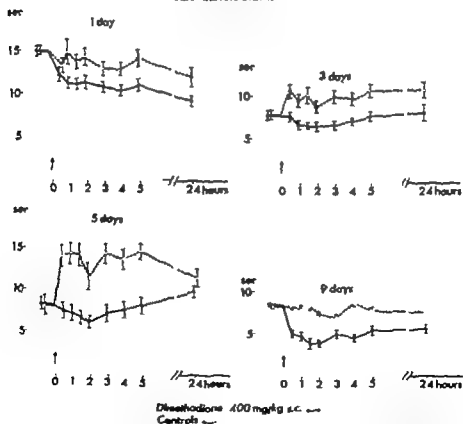


Fig. 7. Mean duration of tonic forelimb seizure in 1 to 9-day-old mice after dimethadione 400 mg/kg subcutaneously. Controls were injected with physiological saline. Vertical bars indicate S.E.M.

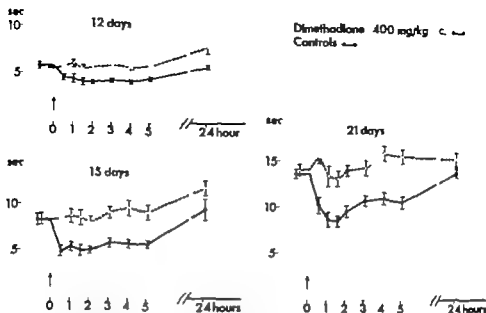


Fig. 8. Mean duration of tonic forelimb seizures in 12 to 21-day-old mice after dimethadione 400 mg/kg subcutaneously. Controls were injected with physiological saline. Vertical bars indicate S.E.M.

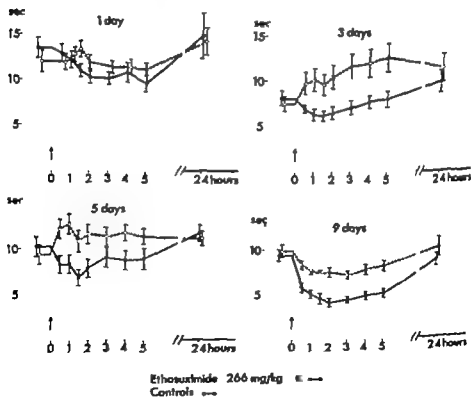


Fig. 9. Mean duration of tonic forelimb seizures in 1 to 9-day-old mice after ethosuximide 266 mg/kg subcutaneously. Controls were injected with physiological saline. Vertical bars indicate S.E.M.

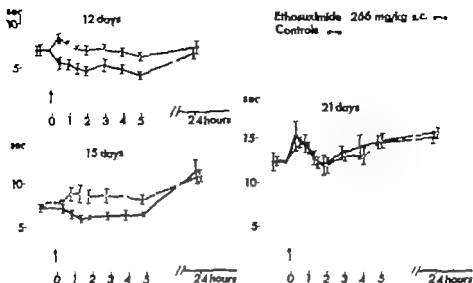


Fig. 10. Mean duration of tonic forelimb seizures in 12 to 21-day-old mice after ethosuximide 266 mg/kg subcutaneously. Controls were injected with physiological saline. Vertical bars indicate S.E.M.

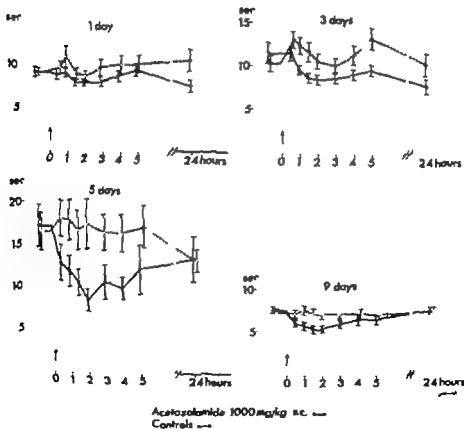


Fig. 11. Mean duration of tonic forelimb seizures in 1 to 9-day-old mice after acetazolamide 1 g/kg subcutaneously. Controls were injected with physiological saline. Vertical bars indicate S.E.M.

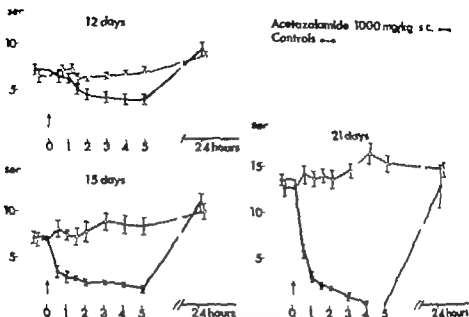


Fig. 12. Mean duration of tonic forelimb seizures in 12 to 21-day-old mice after acetazolamide 1 g/kg subcutaneously. Controls were injected with physiological saline. Vertical bars indicate S.E.M.

of these drugs. It has been shown that the surface area principle for extrapolation of median doses for barbiturates from adult to developing mice gives no correlation with values found experimentally against pentetrazol seizures (FERNGREN 1965 & 1968).

Phenobarbital and mephobarbital are equipotent when tested against conventional electro-shock seizures in adult mice (SWINYARD *et al* 1952). Tested with our electro-shock procedure, mephobarbital seems to be as

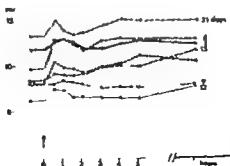


Fig. 13. Mean seizure durations in all saline-injected controls 0.5 to 24 hours after subcutaneous injection. Each point represents values from 70-139 animals. Standard errors of the means are not included in the figure, but are given in table 1 for the pretest values.

Table I

Mean duration of tonic forelimb seizures with the pretest procedure with S.E.M. for the different age groups. The values were compared statistically by Student's *t*-test and *P*-values were given.

Age	n	mean \pm S.E.M	Comparison of age groups	P
1	134	11.82 \pm 0.29	1-3	<0.001
3	90	8.03 \pm 0.27	3-5	0.01-0.001
5	135	9.72 \pm 0.46	5-9	0.01-0.001
9	104	8.10 \pm 0.21	9-12	<0.001
12	82	6.21 \pm 0.17	12-15	<0.001
15	83	8.40 \pm 0.26	15-21	<0.001
21	74	13.31 \pm 0.27	1-21	<0.001

effective as phenobarbital at most ages, but less active in the 9-day-old and the 15-day-old mice and more active in the 3-day-old mice. The duration of effect for both drugs is less than 24 hours in mice over 3 days of age.

Trimethadione and its main metabolite dimethadione have been tested against chemo-shock during maturation and it was found that dimethadione generally had less effect than trimethadione (FERNGREN 1968). Tested against high frequency electro-shock seizures they seem to be equipotent at most ages during postnatal development, but in 15-day-old mice trimethadione has the most marked effect. In adult mice and rats dimethadione has a weaker effect than trimethadione (WITHEROW *et al* 1968) both against chemo-shock (pentetrazol and CO₂) and conventional electro-shock seizures. On the other hand dimethadione is as effective against petit mal in childhood as trimethadione when given in equivalent doses (CHAMBERLAIN *et al* 1965). The duration of the effect is longer for these two drugs than for the barbiturates in 9-day-old and older mice. The effect of ethosuximide against conventional electro-shock seizures is difficult to demonstrate (CHEN *et al* 1963). The same applies to chemo-shock during the postnatal period (Fengren unpublished). In contrast to this an effect against high frequency seizures is observed before 21 days of age. The absence of effect in 21-day-old mice may be related to a very rapid bio-transformation and excretion at that age.

Acetazolamide has a unique structure among the anticonvulsants. Its mode of action is possibly related to inhibition of brain carbonic anhydrase and accumulation of intracellular carbon dioxide. Evidence has

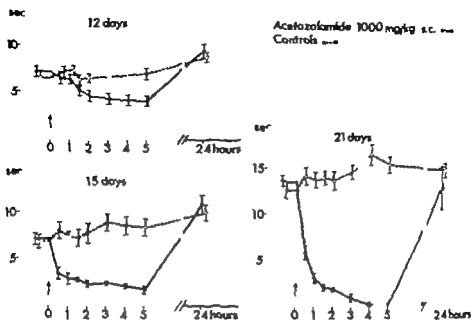


Fig. 12. Mean duration of tonic forelimb seizures in 12 to 21-day-old mice after acetazolamide 1 g/kg subcutaneously. Controls were injected with physiological saline. Vertical bars indicate S.E.M.

of these drugs. It has been shown that the surface area principle for extrapolation of median doses for barbiturates from adult to developing mice gives no correlation with values found experimentally against pentetrazol seizures (FERNGREN 1965 & 1968).

Phenobarbital and mephobarbital are equipotent when tested against conventional electro-shock seizures in adult mice (SWINYARD *et al* 1957). Tested with our electro-shock procedure, mephobarbital seems to be as

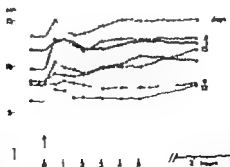


Fig. 13. Mean seizure durations in all saline-injected controls 0.5 to 24 hours after subcutaneous injection. Each point represents values from 70-135 animals. Standard errors of the mean are not included in the figure, but are given in table 1 for the pretreat values.

suximide (266 mg/kg) and acetazolamide (1 000 mg/kg) generally reduced seizure durations significantly as compared to controls. Exceptions were mephobarbital which had no effect in 9-day-old mice and ethosuximide which had no effect in 21-day-old mice. Acetazolamide had a weak effect before the age of 12 days. The duration of the anticonvulsant action was followed for 24 hours and all the drugs showed a longer action in the immediate newborn period, i.e. over 24 hours in 1-day-old mice for all drugs except ethosuximide. Control animals showed an increase in seizure duration with repeated electro-shock. This increase was most marked in 3 and 5-day-old mice and practically absent in 9-day-old mice.

Acknowledgements

The skilful technical assistance of Miss Anna Lisa Paulsson is gratefully acknowledged.

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Effects of Prolonged Chlorpromazine Treatment on the Rat Intestine

By

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(Received November 12, 1968)

Chronic treatment with chlorpromazine (CPZ) is known to cause gastro-intestinal disturbances. It has been demonstrated by means of autoradiographic (IDÄNPÄÄ-HEIKKILÄ *et al.* 1968) and other isotopic techniques (NEUVONEN *et al.* 1968) that it accumulates readily in the intestinal wall of the rat. ZIMMERMAN (1962) has shown that in rats megacolon often develops after the intraperitoneal administration of CPZ.

The purpose of this work was to study the effect of various doses, different routes of administration and duration of CPZ treatment on the development of megacolon. In order to clarify the possible role of 5-hydroxytryptamine (5HT) in this phenomenon, the concentration of 5HT in various parts of intestine was measured after CPZ administration.

Methods

Male Sprague-Dawley rats (initial weight 150-160 g) were used. They were fed with commercial rat pellets (Hankkija Oy Helsinki) and water *ad lib.* The animals received daily for 5 weeks CPZ (chlorpromazine hydrochloride, Medica Oy Helsinki) 20 mg/kg subcutaneously intraperitoneally or by stomach tube, or 5 mg/kg subcutaneously. Some of the animals were given 5 weekly injections of 50 mg/kg CPZ subcutaneously. A group of animals was treated with CPZ 20 mg/kg orally daily for 3 months. The control animals were treated with saline by the same routes as the CPZ-treated rats. CPZ solutions were prepared in saline and the volume injected was always 2 ml/kg. The pH values of the solutions were 5.2, 4.6 and 4.3 respectively to give doses of 5, 20 and 50 mg/kg. Some of the intraperitoneally treated rats were killed 2 months after the last treatment. All the other animals were decapitated 12-18 hrs after the last drug administration. Each experimental group consisted of 4-12 rats.

After killing the animals a 4-5 cm piece of the terminal ileum was removed, as well as the whole of the caecum and the colon down to the rectum. The 5HT concentration was measured by the spectrophotofluorometric method of BOGDANSKI *et al.* (1956). The values given in the text are not corrected according to the recovery of 5HT. The CPZ content was estimated from the same three samples by the spectrophotometric method of SALEMAN & BROOM (1956).

The possible development of lower intestinal dilatation was controlled during the first 5 weeks by an X-ray examination performed every 1-2 weeks after the rectal administration of barium sulphate (micropaque ®, Demancy & Co. Ltd., Slough, Bucks.).

Results

X-ray examination revealed no clear differences between the control and CPZ groups during the first 5 weeks of treatment. In the various animal groups killed after 5 weeks neither intestinal dilatation nor any significant differences in the weights of colon or caecum were observed.

At autopsy of the animals treated intraperitoneally with CPZ (20 mg/kg) for 5 weeks many adhesions could be demonstrated. No dilated areas, however, were seen in the gastro-intestinal tract. Some of the animals ($n = 10$) in this group were not killed until 2 months after discontinuation of treatment. In these rats there were no clear abdominal adhesions, but dilatation of various parts of the gastro-intestinal tract was recorded (fig. 1) in macroscopic examination. Since intestinal dilatation could be recognized in live rats by inspection it became obvious that the major intestinal changes occurred during the second month after discontinuation of CPZ treatment. There were no such gastro-intestinal changes in the experimental groups in which CPZ was given subcutaneously or orally.

The 5HT concentration of the intestine is shown in fig. 2. The doses of 5 and 20 mg/kg of CPZ reduced these concentrations in the caecum after treatment for 5 weeks by about 20 to 60% in the various groups.

After 12 weeks the 5HT content in the animals given 20 mg/kg of CPZ orally was increased in both the caecum and the colon (fig. 3). On the other hand, animals treated with 20 mg/kg of CPZ daily intraperitoneally for 5 weeks and killed after another 2 months showed no significant alterations in the concentration of 5HT in the various parts of the intestine as compared to the controls.

The concentration of CPZ 12-18 hrs after the last injection (fig. 2) was highest in the caecum after 5 and 20 mg/kg given subcutaneously once a day. When 50 mg/kg of CPZ was given weekly subcutaneously the concentrations in the caecum and colon were about the same. In order to study the retention of CPZ in the intestine a single dose of 50 mg/kg was given intraperitoneally. Some CPZ was found in the colon and caecum 24 hrs later but not 3 days later.



Fig. 1 The intestines of rats after i. p. treatment with CPZ (a-c), for details see text. Controls (d).

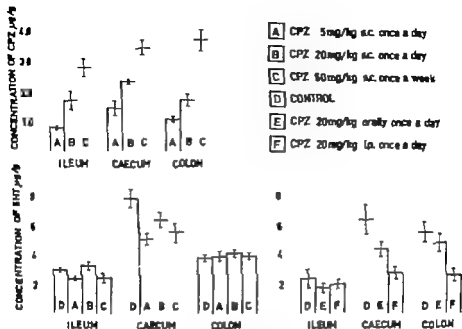


Fig. 2. The concentration of 5HT and CPZ in the wall of the ileum, caecum and colon of rats after various doses and various routes of administration of CPZ for 5 weeks. Means \pm S.E.M. are given, $n = 4 - 14$.

- D CONTROL
- G CPZ 20mg/kg orally once a day
- H CPZ 20mg/kg i.p. once a day

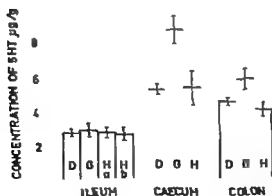


Fig. 3. The concentration of 5HT in the wall of the ileum, caecum and colon of rats after CPZ 20 mg/kg given orally once a day for 12 weeks (G). Another group (H) was treated with CPZ 20 mg/kg i.p. once a day for 5 weeks and killed 8 weeks after discontinuation of treatment. H_a = proximal part, H_b = distal part of the ileum. Means \pm S.E.M. are given, $n = 6 - 12$.

Discussion

The previous finding (ZIMMERMAN 1962) that CPZ can cause megacolon in rats after intraperitoneal injection was studied further in order to analyse the role played by the route of administration of the drug. CPZ can reduce the 5HT content of rabbit blood platelets *in vitro* (BARTHOLOMI *et al* 1961 PAASONEN 1965) and that of the duodenum in acute *in vivo* experiments (ARTER & PAASONEN 1968). Hence the possible role of intestinal 5HT in the development of megacolon was examined.

CPZ treatment in the various doses administered for 5 weeks by different routes did not reveal any dilatation of the intestine, although it decreased the 5HT content of the ileum and caecum even at the smallest dose (5 mg/kg) used. The clearest reduction in 5HT occurred in the caecum, into which CPZ also accumulated most readily. This accumulation of CPZ in the intestinal wall has also been demonstrated previously in autoradiographic studies (SÖSTRAND *et al* 1965 IDÄNPÄÄN-HEIKILÄ *et al* 1968).

If the oral treatment was continued up to 3 months there was still no dilatation of the intestine, and at this time the 5HT was even increased in the caecum and colon. On the other hand, animals treated intraperitoneally for 5 weeks and then given no treatment showed dilatation of various parts of the bowel after another 2 months. However the 5HT content in the dilated parts did not differ from that in the controls. Thus it is obvious that the alterations in the 5HT content are not related to the development of intestinal dilatation.

Moreover the altered reactivity of the colon to various smooth muscle stimulating drugs too does not explain this phenomenon, since in our preliminary *in vitro* studies, the colon of animals treated chronically with CPZ did not differ markedly from the controls in their responses to acetylcholine, nicotine, 5HT and angiotensin. Despite the fact that CPZ in high concentrations inhibits cholinesterases (JOHANNESSEN & LAUSEN 1961 VAPAATALO 1968), the unspecific cholinesterase activity in different parts of the intestine was about the same in the CPZ and control animals observed in our preliminary experiments.

It is obvious from this and previous studies (ZIMMERMAN 1962) that CPZ causes intestinal dilatation only when administered intraperitoneally. The dilatation develops slowly during some weeks. After intraperitoneal administration CPZ causes adhesions possibly due to a local irritation. These adhesions, although not permanent, may explain the results. Another explanation suggested by ZIMMERMAN (1962) could be a high local concentration of the drug in the intestinal wall after intraperitoneal administration. CPZ is known to cause permanent cellular damage in

blood platelets (PAAONEN 1965) and in the adrenal medulla (VAPAATALO 1968)

It is of interest that intestinal dilatation was a more common finding in the autopsy series of mental hospital patients than in the control series (RITAMA *et al* 1969). Although in rats oral CPZ-treatment caused no dilatation of the intestine in 3 months, it is possible that in man, chronic treatment with large doses of major tranquillizers may induce an enlargement of the intestinal volume and even megacolon. This might explain to some extent the gastro-intestinal symptoms observed in psychiatric patients.

Summary

The effect of prolonged chlorpromazine (CPZ) treatment (5 and 20 mg/kg daily for 5 and 12 weeks) given by various routes was studied in rats. An intraperitoneal dose of 20 mg/kg for 5 weeks caused dilatation of the intestine, but this effect was delayed, first appearing 8 weeks after discontinuation of the treatment. The reason for the dilatation may be either adhesions, possibly caused by local irritation by CPZ, or cellular damage due to a high concentration of the drug in the intestinal wall. CPZ did not cause any intestinal dilatation when administered subcutaneously or orally.

The 5HT content of the dilated intestines was not different from that of controls. On the other hand, the treatment for 5 weeks reduced the 5HT content of the caecum, where the CPZ content was also the highest. Oral treatment for 12 weeks somewhat increased the intestinal 5HT content.

Acknowledgements

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Distribution of Inorganic Mercury in the Guinea Pig Brain

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Data on the distribution of toxic substances in the organism have proved useful for assessing the conditions under which such substances may bring about harmful effects. The threshold values for the burden of mercury in various organs during different types of exposure to mercury have been discussed by BERLIN (1963). In an autoradiographic study in the mouse, he showed that mercury is accumulated in the organs that are "critical" in mercury poisoning. It seems that mercury is metabolized more slowly in the brain than in most other organs (BERLIN 1963 FRIBERG 1956 and others). The brain is also a critical organ in prolonged exposure to mercury vapor. The fact that an organ is "critical" however is due to the concentration in certain of its cells reaching a level at which cell damage arises, rather than to the mean concentration in the organ reaching a particular level. Since there is a greater similarity between the cells of a particular organ than between those from different organs in that the sensitivity of the former to e.g. mercury will be more uniform, the finding of major variations in the concentration within an organ can be expected to indicate which cells in the organ run the greatest risk of being injured. BERLIN's whole-body autoradiographic studies on the mouse point to the existence of concentration differences in the central nervous system following the injection of inorganic mercury.

The present paper concerns the first part of a study on the distribution of mercury in the central nervous system. The aim was to determine whether the concentration differences in the brain are ascribable to the uptake of mercury in certain cells or whether the concentration gradients are primarily determined by other factors such as the blood circulation

in the area, the proximity of blood vessels, or the cerebrospinal fluid, etc. We also wished to investigate whether the rate of elimination varies between different areas of the central nervous system, since this might be relevant to the question of whether prolonged exposure is liable to elicit different types of injury as compared to short term exposure.

The uptake of mercury in the brain is about ten times greater when mercury vapor is inhaled than when a solution of mercury salt is administered intravenously (BERLIN JERSEKELL & V UBISCH 1966). As far as the distribution within the brain after mercury inhalation is concerned, however it is still not clear whether the greater uptake in this organ reflects a generally increased entry resulting in the same pattern of distribution, or an increased uptake in particular structures, resulting in a different distribution pattern.

Autoradiography appears to be the simplest means of investigating these problems concerning the distribution of mercury in the brain. Previous autoradiographic studies in the mouse gave only a rough picture of the distribution of mercury in the central nervous system and consequently it was decided instead to perform the experiments on the guinea pig. Compared with the mouse the size of this animal allows of a substantially better autoradiographic resolution of different brain structures.

The present study dealt with a comparison between guinea pigs exposed to radioactive mercury vapor and guinea pigs injected with a radioactive solution of mercuric nitrate.

The total amount of mercury in the animal and the contents in various organs were determined by scintillation counting in order to see whether the guinea pig also displays an increased uptake of mercury in the brain after inhalation.

Material and Methods

The experiments were performed on 25 guinea pigs with average weight of 300 g. Radioactive metallic mercury was obtained from ^{203}HgO (supplied by AB Atomenergi, Stockholm, Sweden, specific activity 3 ci per gram Hg) by dissolving in nitric acid and separation by electrolysis as previously described (BERLIN, JERSEKELL & V UBISCH 1966). A mercuric nitrate solution, containing 0.26 mg mercury/ml after neutralization was used for injection.

Experimental design

One series of 13 guinea pigs was exposed for 3 hours to a concentration of 7 mg radioactive mercury vapor per m^3 air after which the animals were sacrificed in groups of 3 (4 in the acute group) immediately, 24 hours and 6 and 11 days later. In another series, 1 guinea pig received intravenous injections of radioactive mercuric nitrate solution and was sacrificed in groups of 3 after intervals of 4 hours, 24 hours, 6 days and 16 days.

The exposure to radioactive mercury vapor was undertaken in the apparatus described

by BERLIN, NORDBERG & SÄRÄNEN (1969). The injection of the radioactive mercury was achieved by administering the solution of mercuric nitrate to the animal intravenously in a limb vein exposed under sterile conditions. The injected dose corresponded to 0.4 mg mercury/kg body weight. Whole-body counting was used to check that the entire dose was taken up in the animal. The animals were sacrificed with an overdose of pentobarbital (Abbot) intraperitoneally. Blood samples were obtained by puncturing the jugular vein during this anesthesia, just before cardiac arrest.

Whole-body counting

Immediately after exposure the animal was measured in a defined geometrical position between two sodium iodide crystals. These measurements were then related to those for a standard of the same shape as the animal, measured in the same way. Since the standard contained a known amount of the radioactive isotope used, it was easy to calculate the amount of mercury taken up in the exposed and injected animals respectively.

Organ assay

Samples of blood, brain, liver, cardiac muscle, kidney and lung were measured in a well crystal against a standard, making it possible to calculate the amount of mercury in the sample in the same way as above. In all cases the radioactivity in the sample was several times greater than the background level.

Autoradiography

As soon as the animal had been sacrificed, the brain was removed and divided down the midline. One half was used for the organ measurements referred to above. The other half was frozen in a refrigeration mixture consisting of carbon dioxide and hexane (-80°C), embedded in carboxy-methylcellulose and then sectioned at intervals of 0.5–1 mm to give longitudinal sections 15 μ thick on tape (Scotch tape no. 810) (unpublished, in manuscript). Autoradiography was then performed on Structurix (Geaver) radiographic film according to the method described by Ullberg. The exposure time varied between 6 and 30 days. The autoradiograms were then compared with the histological sections, which were stained with toluidine blue to give good differentiation between gray and white matter. A standard staircase was included in the autoradiography of each section (according to BERLIN & ULLBERG 1963), thus allowing semiquantitative evaluation of the differences in concentration between different parts of the brain.

Results

The results of the scintillation counting are given in table 1. The mean concentration of mercury in the brain is 3–9 times higher in the inhalation animals than in the injected animals. The lungs of the animals exposed to mercury vapor naturally contains considerably higher initial quantities of mercury than the lungs of the injected animals. The measurements of other organs do not show any consistent differences between the different groups.

The autoradiograms of the brain sections show a differentiated picture. Figs. 1 and 2 illustrate how the distribution in the brain changes with the

Table I

The concentration of mercury in different tissues expressed as % of initial whole body burden of mercury per gramme of tissue.
A. Vapor exposed animals.

Survival time	Lung		Blood		Brain		Liver		Myocardium		Kidney	
	Individual values	Mean	Individual values	Mean	Individual values	Mean	Individual values	Mean	Individual values	Mean	Individual values	Mean
Acute.	5.29		0.274		0.153		0.210		0.458		4.80	
Acute	7.15	6.34	0.226		0.121		0.147		0.361		5.59	
Acute	7.46		0.328		0.150	0.162	0.351	0.289	0.727	0.351	8.25	5.89
Acute	5.45		0.277		0.222		0.446		0.536		4.94	
24 hours	1.46		0.592		0.406		0.747		0.550		20.67	
4 hours	2.14	1.80	0.129		0.116	0.211	0.206	0.361	0.155	0.278	6.49	10.88
24 hours	1.80		0.129		0.110		0.129		0.129		5.48	
6 days	1.05		0.033		0.100		0.081		0.041		5.97	
6 d. ys	1.60	1.17	0.043		0.099	0.105	0.350	0.250	0.047	0.045	5.56	6.05
6 days	0.85		0.027		0.116		0.318		0.047		6.62	
16 days	0.027		0.0025		0.040		0.046		0.0031		1.09	
16 days	0.017	0.032	0.0032		0.018	0.032	0.037	0.044	0.0043	0.0065	1.10	1.31
16 days	0.052		0.0040		0.039		0.050		0.0098		1.74	

B. Injected animals.

Survival time	Lung		Blood		Brain		Liver		Myocardium		Kidney	
	Individual values	Mean	Individual values	Mean	Individual values	Mean	Individual values	Mean	Individual values	Mean	Individual values	Mean
4 hours	0.308		0.114		0.0395		0.834		0.129		9.50	
4 hours	0.336	0.575	0.279	0.197	0.0312	0.0442	1.897	1.219	0.166	0.161	10.40	9.92
4 hours	0.441				0.0430		0.926		0.186		9.86	
24 hours	0.445		0.1327		0.0279		0.721		0.1165		6.91	
24 hours	0.375	0.404	0.0486	0.0875	0.0152	0.0742	0.992	0.593	0.0937	0.0991	7.7	7.88
24 hours	0.352		0.0411		0.0204		0.466		0.0811		9.031	
6 days	0.149		0.0493		0.0590		0.274		0.0406		4.76	
6 days	0.229	0.141	0.0440	0.0366	0.0267	0.0320	0.124	0.174	0.0608	0.0384	4.24	4.79
6 days	0.045		0.0165		0.0113		0.125		0.0137		5.37	
16 days	0.0208		-		0.00518		0.046		0.00453		1.75	
16 days	0.0727	0.0359	0.0118	0.0184	0.01686	0.00491	0.199	0.101	0.01420	0.00737	1.70	1.86
16 days	0.0140		0.0249		0.00468		0.037		0.00342		2.13	



Fig. 1 Autoradiograms of guinea pig brains at various survival times after an injection of radioactive mercuric nitrate solution. *a*, after 4 hours; *b*, after 24 hours; *c*, after 6 days; *d*, after 16 days.

Interval after injection and the exposure to mercury vapor respectively. Both types of administration give the same general picture. The autoradiograms thus have a patchy appearance immediately after exposure or injection though this patchiness is considerably more marked in the inhalation than in the injected animals. After 24 hours there is in principle

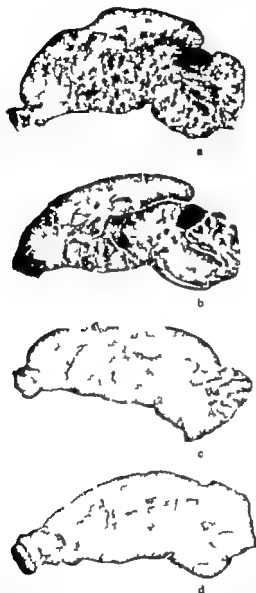


Fig. 2 Autoradiograms of guinea pig brains at various survival times after inhalation of radioactive mercury vapor *a*, after 0 hours *b* after 4 hours; after 6 days *c*, after 16 days.

a more marked uptake of mercury in the grey than in white matter and this pattern (though less pronounced) is also visible after 6 days. After 16 days, the pattern of distribution has changed, the concentration of mercury in the grey matter being considerably reduced in e.g. the cerebral cortex, but still relatively high in other regions. Consequently the corpus

callosum gives a blacker picture than the surrounding cerebral cortex at this time.

Although the general pattern of distribution is much the same for both methods of administration, certain differences were noted. As already mentioned there were also considerable differences in the uptake and elimination of mercury between different regions of the brain. The distribution in different parts of the brain is therefore briefly described below (using the nomenclature used by CRAIGIE *et al* 1963)

Rhombencephalon.

In the rostral part of the medulla oblongata the nuclei - olivary nucleus (figs. 1d & 2b) etc. - appear blacker than the tracts at all times. As already mentioned the acute stage after inhalation is characterized by a patchiness that appears to be located around the blood vessels, though here too there is in principle a greater uptake in the grey than in white matter. A very pronounced blackening in the area postrema was observed in the injected animals but not in those that inhaled the mercury (figs. 1d & 6). This high concentration seems to have remained unchanged during the period of observation and consequently the area postrema contrasts most markedly with its surroundings 16 days after the injection (fig. 6).

Within the pons, the relationship between the grey and white matter is similar to that described for the medulla oblongata for survival times up to 6 days. However the mercury is eliminated more slowly from the tegmentum than from the neighbouring white matter and consequently the former appears more clearly on autoradiograms after 16 days (figs. 1d & 3). The nuclei pontis are also visible (figs. 1c, 1d, 2c, 2d & 3).

The cerebellum presents high concentrations in the granular layer (figs. 1a, 1b & 2b) and in the central nuclei (nucleus dentatus etc., fig. 1a) on autoradiograms from the short survival times, while the molecular layer and white matter contain lower concentrations. As in the cerebrum, the distribution pattern in the cerebellum changes with time as the mercury is eliminated more rapidly from the cortex than from the medulla. After 16 days the concentration in the white matter is consequently the same as in the granular layer while the molecular layer produces practically no blackening of the radiogram. Mercury appears to be eliminated slowly from the nucleus dentatus, which thus shows up strikingly on autoradiograms after 16 days.

Mesencephalon

The main feature of interest here is the very marked accumulation of mercury in a couple of nuclear areas situated dorsally and medially near

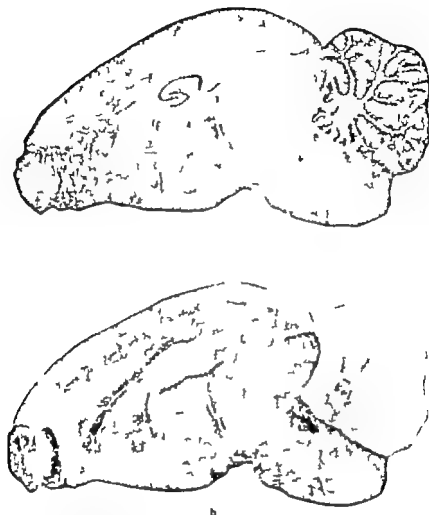


Fig. 3 a, Frozen section ($\times 4$ toluidine blue) of guinea pig brain 18 days after inhalation of radioactive mercury vapor. b, Autoradiogram of the same section.

the borderline to the rhombencephalon, and roughly adjoining the nucleus oculomotorius and nucleus trochlearis (figs. 3 4 & 5). These nuclei appear very black on autoradiograms from injected animals (fig. 5) as well as from inhalation animals (figs. 3 & 4). No elimination of mercury was observed from these structures and they are consequently most marked on autoradiograms after 16 days (figs. 3 4 & 5). The colliculi posteriores also produce heavy blackening, particularly at the short survival times (figs. 2a & 2b).



a



b

Fig. 4. a, Part of the mesencephalon in the section reproduced in fig. 3 ($\times 20$). b, Autoradiogram of the same part.

Diencephalon

This shows little differentiation, though a relatively marked accumulation was observed in the nucleus habenularis in the epithalamus on some autoradiograms. In the hypothalamus, the tuber cinereum presents a higher activity than its surroundings. The elimination of mercury was relatively rapid from the diencephalon, with the exception of the structures mentioned above and a thin layer bordering on the ventricles (ependymal cells) cf figs. 1c, 1d & 2d

Telencephalon

As already mentioned the concentration in the cerebral cortex is high after the short survival times, and considerably higher than in e.g. the

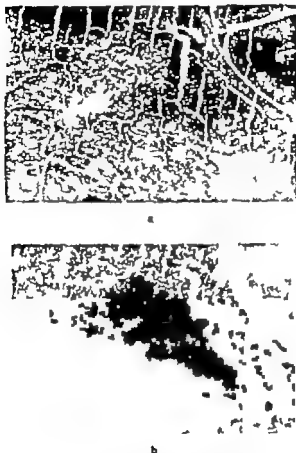


Fig. 5. a, Part of the mesencephalon from the brain of guinea pig sacrificed 16 days after an injection of radioactive mercury nitrate solution ($\times 20$, toluidine blue) b, Autoradiogram from the same part.

corpus callosum. Since the mercury is eliminated rapidly from the cortex, however the corpus callosum is much blacker than the cerebral cortex on autoradiograms after 16 days. In some autoradiograms a lamination in the cerebral cortex could be observed (fig. 2b & 2c) indicating that mercury is taken up by the nerve cells. The limbic pathways display the same slow elimination as the corpus callosum and are thus clearly visible after 16 days (figs. 1c, 1d & 3). The hippocampus does not appear to take up inorganic mercury to any appreciable extent. Moreover the concentration in this structure diminishes at least as rapidly as in e.g. the cerebral cortex. This is also generally true of other structures in the telencephalon, though certain parts of the olfactory bulb accumulate mercury

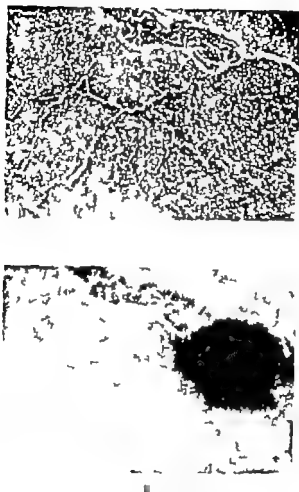


Fig. 6. *a*, Section ($\times 20$) from guinea pig brain 16 days after injection of radioactive mercuric nitrate solution, showing the plexus chorioideus in the upper part of the section and area postrema to the left. *b*, Autoradiogram of the same section.

The plexus chorioideus is a prominent feature in the injected animals at all survival times (figs. 1 & 6) though the concentration in this structure decreases at the same rate as in other parts of the brain. In the inhalation animals, on the other hand, the structure is not prominent at any survival time (fig. 2).

The above differences in blackening on the autoradiograms were estimated semiquantitatively with the help of a "standard staircase". It was found that the difference in concentration between the most strongly and weakly blackened parts of e.g. the autoradiograms reproduced in figs. 1d and 3 amounts to a factor of more than 2^8 ($= 256$).

The autoradiograms from different animals with the same survival time usually showed a good conformity concerning the distribution pattern. It should be noted, however, that certain individual differences were observed in the distribution pattern after 16 days, particularly in the injected animals. Autoradiograms representing an intermediate stage between the 8 and 16-day autoradiograms reproduced in fig. 1 were thus obtained from guinea pigs injected with a solution of mercuric nitrate. There were also relatively large individual differences in the scintillation counting (table 1) and it was found that the animals with the lower contents of mercury in the brain (according to the scintillation counts) displayed the picture illustrated in fig. 1d.

Discussion

Previous investigations on the mouse have shown that the administration of mercury by the inhalation of the vapor results in a considerably higher concentration of this substance in the brain than the injection of a solution of inorganic mercury salt. Subsequent studies by our research team showed that this was also true of the rat, rabbit, and monkey (BERLIN FAZACKERLY & NORDBERG 1969). The present investigation has demonstrated a similar difference in the guinea pig, justifying the use of this animal for studying the problem outlined in the introduction, namely the accumulation and distribution in the brain of the greater amounts of mercury in the inhalation animals.

The high concentration in the plexus choroideus and area postrema in relation to surrounding tissue after injection and the more uniform distribution of mercury in the acute stage suggest that the uptake mechanisms involved after injection may differ somewhat from those after inhalation. The patchiness in the acute stage after inhalation may well reflect a direct transfer of mercury from the blood to the cerebral tissue. A number of guinea pigs exposed to mercury vapor for 10 minutes had the same percentage of mercury in the brain as was measured in the present study after exposure to this vapor for 5 hours (NORDBERG unpublished). This was no doubt partly because the concentration of mercury in the blood immediately after 10 minutes exposure was approximately twice as high (as a percentage of the whole-body count) as after exposure for 5 hours though the finding nevertheless suggests that a substantial amount of mercury had penetrated into the cerebral tissue after only 10 minutes. This observation reinforces the assumption that mercury administered by the inhalation of vapor is transferred directly from blood to brain tissue.

With the exception of the plexus chorioideus and area postrema, however the pattern of distribution is much the same for the two methods of administration, so that the same structures in the brain contain mercury. There is thus no evidence to show that the higher concentration in the brain of inhalation animals reflects a selective increase in special structures.

The present study clearly demonstrated very considerable differences (exceeding a factor of $2^8 = 256$) between the concentrations in different parts of the brain. It also showed that mercury is eliminated at different rates from different structures.

Except for the acute stage, the distribution of mercury in the brain does not seem to be related to the blood vessels. The distribution appears to be related to the relative affinity of the various cells for mercury. This is quite evident for some regions where a selective uptake of mercury takes place in cells with a particularly high affinity for this substance. In some cases these cells have a mercury concentration that is more than $2^6 (= 64)$ times that in neighbouring cells. These mercuriophil cells in the mesencephalon also display a marked capacity for retaining the mercury and consequently the concentration gradient between these and neighbouring cells becomes larger and larger with increasing survival times after administration of mercury. It therefore seems reasonable to expect a marked accumulation in these cells in animals subjected to prolonged exposure to mercury. While these cells are probably not substantially less sensitive to mercury than other cells, it is not yet possible to say anything definite about the toxicological importance of this selective accumulation.

An interesting question is whether a similar marked uptake of mercury is shown by corresponding cells in the brains of other animals and also in man. The distribution of mercury in the brain of the monkey is being studied at present in this laboratory and certain results are awaiting publication elsewhere. In principle the distribution of mercury in the monkey appears to resemble that reported here for the guinea pig. The highly mercuriophil cells in the mesencephalon have not, however been detected as yet, possibly because the brain stem in primates constitutes a relatively smaller part of the brain than in rodents. More detailed studies on the monkeys are now in preparation.

As a result of the differences in the rate at which mercury is eliminated from different parts of the brain the distribution pattern 16 days after administration of mercury is partly the reverse of that found after 24 hours. If this development is repeated for each individual dose during prolonged exposure, the highest mercury burden in the present investigation will be found in the structures that display the highest concentrations after 16 days. This would mean that conditions exist for the development of selective injury in these regions of the brain.

Summary

Groups of guinea pigs were exposed to radioactive mercury vapor or given intravenous injections of radioactive mercuric nitrate solution, after which the animals were sacrificed at various intervals up to 16 days after the injection or the exposure to mercury vapor. The amount of mercury in the brain was determined by scintillation counting and the distribution within the brain was illustrated autoradiographically.

The uptake of mercury in the brain proved to be several times greater in the vapor exposed animals than in the injected animals. The distribution of mercury in the brain, however, was similar after both types of administration. The elimination of mercury is different from different parts of the brain and consequently the distribution pattern, a long time after the administration of mercury, is not the same as that found immediately afterwards. Substantial differences were found between the concentrations in different parts of the brain: certain cells in the brain stem still contained particularly high concentrations long after the administration of mercury.

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Kwashiorkorigenic Diet and Diazinon Toxicity

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The objective of the project described in this report was to find the effect of a low protein diet on the acute oral toxicity of diazinon in albino rats. When weanling albino rats are fed a protein-deficient diet, they develop a syndrome similar to that of kwashiorkor (DE CASTRO & BOYD 1963). Kwashiorkor is basically a manifestation of the effects of deficient intake of protein by human infants and is most prevalent in those parts of the world where the diet is low in proteins (WHO REPORT 1965). It was anticipated, therefore, that the results of the present study would have some application to the use of diazinon as a pesticide in countries where the diet is low in protein.

Diazinon is a widely used pesticide of the class of organophosphate cholinesterase inhibitors. It is a derivative of diethyl pyrimidinyl phosphorothionate which was first synthesized in 1952 (SPENCER 1968). It is a colourless liquid when pure the technical grade (91-92%) is a pale brownish liquid with a faint musty odour which we found to be insoluble in water but soluble in cottonseed oil. Diazinon is efficiently absorbed by the mammalian gastrointestinal tract, partially metabolized by hydrolysis and oxidation and rapidly eliminated in the urine (FAO/WHO REPORT 1965). Residues on crops (GUNTHER *et al* 1958) and soil (MALONE *et al* 1967) are relatively short lived and tolerance limits in foods have been placed at 0.1 to 0.75 p.p.m. in man (CHAPMAN 1967) and 2 p.p.m. in rats (FAO/WHO REPORT 1967).

Experimental Methods

The experiments were performed on 421 male albino rats of Wistar strain obtained from Canadian Breeding Laboratories of St. Constant, Quebec, Canada. They were divided into three dietary groups, group (a) fed Purina laboratory chow obtained from the Ralston

Purina Company of Woodstock, Ontario, group (b) fed Protein Test Diet, Normal, obtained from General Biochemicals, Chagrin Falls, Ohio U.S.A., and group (c) fed Protein Test Diet, Low also obtained from General Biochemicals.

Animals in groups (b) and (c) were purchased as weanlings weighing 50 to 60 g and were fed their respective diets for 28 days, after which they were given diazinon. De Castro & Boyd (1968) have shown that kwashiorkor-like inhibition of growth reaches steady state after 2 weeks in weanlings fed a protein-deficient diet and 28 days was therefore selected as a satisfactory feeding interval. Animals of group (a) were purchased as 4-week old male rats and were fed laboratory chow until their body weight was 5 to 10 percent higher than that reached at 28 days in animals of group (b) and then given diazinon. De Castro & Boyd (1968) reported that weanlings fed laboratory chow for 28 days weighed 5 to 10 percent more than corresponding weanlings fed Protein Test Diet, Normal.

Protein Test Diet, Low contains 3.47% casein, 81.53% cornstarch, 8.0% hydrogenated cottonseed oil, 4.0% salt mix (U.S.P. XIV), and 3.0% of an all-vitamin supplement prepared according to Hegsted & Crane (1965). This diet was selected because Boyd & De Castro (1968b) found it contained the highest amount of casein which would produce definite kwashiorkor-like syndrome when fed to weanling albino rats. Protein Test Diet, Normal, contains 26.0 percent casein and 59.0 percent cornstarch but otherwise has the same composition as Protein Test Diet, Low. It was used to determine any effect of the purified diet other than protein deficiency. From the studies of Boyd & Lru (1968) it was concluded that differences in the starch content of diets fed to groups (b) and (c) would have insignificant effects.

Each rat was weighed and placed singly in metabolism cage for 16 hours with water but no food (i.e. empty the stomach) before the administration of diazinon. Diazinon was used as Diazinon Technical, 91.4% diazinon, ARS No. 235/68, Batch No. FL-6199 supplied by Dr. Joseph Marrus of Geigy Agricultural Chemicals, Division of Geigy Chemical Corporation, Ardsley, New York. It was dissolved in cottonseed oil and was given by intragastric cannula in a constant volume of 20 ml/kg body weight. Following pilot studies to estimate the range of the LD₅₀, diazinon was given in doses of from 50 to 700 mg/kg, 10 rats per dose, while from 15 to 45 control animals were given cottonseed oil with no dissolved diazinon.

Following the administration of diazinon the animal was returned to its metabolism cage and given a weighed amount of laboratory chow (50 g) and measured volume of tap water (100 ml). Observations of any clinical signs were made hourly during the day of drug administration and then at intervals of 4 hours after the administration of diazinon. Clinical signs were semi-quantitated in clinical units of 1 to 4. At intervals of 24 hours for 4 days, when the toxicity syndrome had subsided, the body weight was measured as g gain (or loss) per day. Food intake was determined as g/kg body weight per 24 hours, water intake as ml/kg/24 hours, colonic temperature as degrees centigrade, urinary volume as ml/kg/24 hours, urinary glucose and protein output as mg/kg/24 hours, urinary blood as units/kg/24 hours, and urinary pH on the 24-hour sample. Premortem signs were recorded in detail on any rat which could be closely observed at the time of death. Details of method have been recently reviewed by Boyd (1968).

All animals which died were autopsied and the gross pathology recorded. When autopsy could be performed immediately after death, microscopic examinations were performed on any organ which appeared grossly abnormal. In addition, histopathology in representative animals was recorded on all organs listed in table 2. For microscopic examination, blocks of tissue were fixed in Lillie buffered formalin and sections were stained with haematoxylin-phloxine-saffron.

The fresh wet weight and water content of organs listed in tables 3 and 4 were determined

in rats of group () which died and could be autopsied within 1 hour of death in order to avoid postmortem shifts described by BOYD & KNOTT (1963). Similar measurements were made on survivors at 2 weeks and 1 month and on controls (given cottonseed oil only) at corresponding periods. Survivors and controls were killed by inhalation of chloroform since intracranial injection of ethanol, recommended by PREIFFER & MULLER (1967) precluded the desired observations on the brain. The numbers of animals used in making these measurements are indicated in tables 3 and 4. Organ weights and water levels were not determined in rats of groups (b) and (c) since pilot studies had indicated that the differences from controls were the same as in rats of group (a).

Organ weights were determined to 0.1 mg on a Mettler semimicrogrammatic balance except for the skin and residual carcass which were weighed to 0.01 g. The weight of the gastrointestinal organs was measured after removing the contents of the lumen by a standardized process of water washing, milking, and filter-paper drying. The sample of skeletal muscle was the right half of the muscular layer of the ventral abdominal wall. The brain included the medulla oblongata. The sample of skin included the tail. The residual carcass was weighed, cut into small pieces and homogenized in a Waring blender.

Water content was determined on weighed aliquots which were then dried to constant weight at 95 °C in a Fisher forced-draft Isotemp (temperature-controlled) oven. The sample of skin for water analysis was taken from the dorsolumbar region after clipping off the hair. The sample of residual carcass was from the homogenate of the residual carcass. Water content was calculated as g water per 100 g dry weight of tissue.

The results were analyzed statistically by subjecting the differences between the respective means to *t*-tests of significance and by regression of mean differences on dose or time, using methods described by GOUTMAN (1952). The median lethal dose (LD_{50}) \pm S.E. was calculated by the linear regression method of BOYD (1965).

The LD_{50} in rats of group () was found to be appreciably higher than the values reported in the literature and reviewed by the FAO/WHO REPORT (1965) and by SHOWN (1968). Enquiries to the manufacturer of diazinon disclosed that the batch of Diazinon Technical which was provided for this study had been stabilized by a new process. A supply of Diazinon Technical stabilized by the older process was provided to us under the designation Diazinon Unstabilized Stabilized in Ardsley ARS No 1613/68, Batch N R3-102401 (FL 8722) and its acute oral LD_{50} \pm S.E. was determined in the rats of group (a).

Results

Group (a) Fed Laboratory Chow The $LD_{50} \pm$ S.E. was found to be 466 ± 87 mg/kg, the maximal LD_0 was estimated at 161 mg/kg and the minimal LD_{100} at 770 mg/kg. The mean \pm S.D. interval to death in the range of the LD_{50} was 23 ± 8 hours, the interval being shorter the higher the dose. Death occurred in deep hypothermic coma following respiratory failure.

The clinical signs observed in the hours immediately following administration of diazinon included listlessness, drowsiness, diarrhoea tremors, tachypnoea ataxia, exophthalmos, sialorrhoea dacryorrhoea, and epistaxis. The intensity of these signs increased with increasing dose of diazinon. At 24 hours, there was marked drowsiness prostration, dyspnoea,

Table 1

Representative changes in clinical measurements at 24 and 72 hours after oral administration of diazinon (doses in the range of the LD₅₀.)

Measurement	At 24 hours			At 72 hours		
	Group ()	Group (b)	Group (c)	Group (a)	Group (b)	Group (c)
Gai in body weight	-25.7)	-19.2)	-12.7)	+31.6)	-6.5)	+ 6)
Food intake	- 34.1)	- 79)	- 57)	- 9	-40)	+ 19
Water intake	+ 3.2	- 52)	- 80)	+ 17)	- 3	+ 20)
Colonic temperature	- 2.3)	- 6.7)	- 2.0)	+ 0.3	- 0.3	+ 0.8)
Urinary volume	+101)	- 35.2)	- 8.3)	+ 69)	- 4.3)	+111 3)
Urinary glucose output	+173)	+139)	+ 53.2)	+167)	+ 2.1)	- 65.3)
Urinary protein output	+ 1.7	- 20.8	- 15.6	+ 33)	+12.2	+155)
Urinary pH	+ 7.2)	- 3.3)	- 12.6)	+ 4.3)	- 6.4)	- 4.1
Urinary blood ²⁾	+ 1.3)	+ 3.5)	+ 1.5)	+ 2.6)	0.0)	0.0)

1) The results are expressed as mean percent change from controls fed the same diet and given only cottonseed oil, specifically as $((\bar{X}_d - \bar{X}_c)/\bar{X}_c) \times 100$ where \bar{X}_d is the mean in diazinon-treated rats and \bar{X}_c is corresponding controls.

2) Expressed $\bar{X}_d - \bar{X}_c$ since \bar{X}_c was usually zero.

3) $\bar{X}_d - \bar{X}_c$ was significantly different from zero at $P = 0.02$ or less.

4) Significantly different from Group (a) at $P = 0.02$ or less.

5) Significantly different from Group (b) at $P = 0.02$ or less.

exophthalmos and ataxia apparently due in part to a fall in body temperature.

Clinical measurements at 24 hours are summarized in table 1. There was a loss of body weight due to the diarrhoea, to decreased food intake and to a diuresis. This was accompanied by a fall in colonic temperature, a glycosuria, alkalmuria, and haematuria. These signs were dose-dependent.

Gross and microscopic observations on body organs at autopsy are summarized in table 2. There was a local inflammatory reaction in the gastrointestinal tract, a stress reaction in the adrenal glands, spleen and thymus gland capillary-venous congestion in several organs and occasionally broncho-pneumonia.

Shifts in organ weights are summarized in table 3. Loss of body weight was found to be mainly due to loss of muscle weight. The caecum, liver spleen, and thymus gland also showed loss of weight.

Death was found to be accompanied by a more or less generalized dehydration of body organs as indicated in table 4. The only organs which were significantly hydrated were the adrenal glands and the liver

Table 2

Gross and histopathological observations at autopsy on albino rats which died from oral administration of diazepam.¹⁾

Organ	Gross pathology	Histopathology
Adrenal glands.	11 % red	Lipoid granules prominent occasionally vacuolation, especially in the zona fasciculata.
Brain	Normal	Normal appearance.
Gastrointestinal tract		
Cardiac stomach	Normal	Normal appearance.
Pyloric stomach	50 % ulcers	Lamina propria congested with occasional small areas of haemorrhage and necrosis at the mouth of the gastric glands.
Small bowel	34 % inflamed	Lamina propria occasionally congested.
Caecum	12 % inflamed	Lamina propria occasionally congested.
Colon	Normal	Normal appearance.
Heart	Normal	Occasionally capillary congestion.
Kidneys	Normal	Tabular cloudy swelling capillary congestion of the loop region.
Liver	20 % dark	Occasionally sinusoidal congestion.
Lungs	33 % inflamed	Pulmonary vascular congestion, venous stasis and occasionally extensive pneumonia.
Muscle (ventral abdominal wall)	Normal	Normal appearance.
Salivary (submaxillary) glands	Normal	Deficiency of zymogenic granules in the serous glands.
Skin	Normal	Normal appearance.
Spleen	Small	Red pulp contracted
Testes	Normal	Normal appearance.
Thymus gland	Small	Varying degrees of atrophy from minor to almost complete loss of thymocytes.

¹⁾ Rats of group (c) exhibited, in addition, signs due to marked protein deficiency namely impaired maturation of the intestinal mucosa, skeletal muscle, pancreas, salivary glands, testes and thymus gland, fatty degeneration in the liver and lesions of atrophic dermatitis in the tail (see Boyd & De Castro 1968a)

Recovery was rapid in the survivors. At 48 hours, the daily growth rate had returned to normal and by 72 hours this was in excess of that in the controls (see table 1) There was some polydipsia and the colonic temper

Table 3

Changes in the fresh wet weight of body organs of albino rats following oral administration of diazinon in doses in the range of the LD₅₀.¹⁾ The animals were previously fed laboratory chow

Organ	At death (N = 10 plus 14 controls)	Survivors at 2 weeks (N = 15 plus 15 controls)	Survivors at 1 month (N = 15 plus 14 controls)
Adrenal glands	+ 4.8	-4.2	+20.0
Brain	+ 3.2	-4.4	+ 1.3
Gastrointestinal tract:			
Cardiac stomach	+ 1.3	-3.6	+13.3
Pyloric stomach	+ 2.3	+0.1	+ 3.8
Small bowel	+ 7.8	+3.1	+ 2.2
Caecum	-31.8	-0.1	+ 8.6
Colon	+ 9.8	+0.3	- 1.7
Heart	+ 3.4	+3.2	+ 6.9
Kidneys	- 0.2	-0.3	+ 7.0
Liver	- 6.2	+5.2	+11.5
Lungs	- 2.1	-3.8	+ 6.6
Muscle (ventral abdominal wall)	-13.9	+8.9	+11.8
Salivary (submaxillary) glands	+ 3.2	+2.3	+ 0.2
Skin	+ 4.1	+3.9	+10.9
Spleen	-35.0	+6.1	+30.1
Testes	- 1.8	+0.5	- 1.2
Thyroid gland	-28.5	-0.7	+19.2
Residual carcass	- 8.2	+4.7	+13.1
Body weight	- 8.8	+4.4	+12.3

¹⁾ Fresh wet weight was measured in grams. The results are expressed as mean percent change from controls, specifically as $(\bar{X}_d - \bar{X}_c/\bar{X}_c)$ where \bar{X}_d is the mean in the diazinon-treated animals and \bar{X}_c the corresponding mean in the controls. One asterisk indicates that $\bar{X}_d - \bar{X}_c$ was significant at $P = 0.05$ to 0.02 and two asterisks at $P = 0.01$ or less.

ature was normal at 72 hours. The diuresis, glycosuria, proteinuria, alkalimuria, and haematuria were still present at 3 days however. By 2 weeks most of the organ weights (table 3) and water levels (table 4) had returned to values found in the controls. By 1 month most of the organs in the survivors weighed more than those in the controls (table 3) this being due to an increase in dry weight since the organ water levels tended to be slightly below those in the controls (table 4).

Group (b) Fed Protein Test Diet Normal. During the 4 weeks of feeding there were 15 deaths out of a total of 110 rats placed on diet, a dietary

Table 4

Changes in the water content of body organs of albino rats following oral administration of diazinon in doses in the range of the LD₅₀.¹⁾ The animals were previously fed laboratory chow

Organ	At death (N = 10 plus 14 controls)	Survivors at 2 weeks (N = 15 plus 15 controls)	Survivors at 1 month (N = 15 plus 14 controls)
Adrenal glands	+23.3	-14.2	-3.4
Brain	+ 3.2	+ 0.4	-3.0
Gastrointestinal tract			
Cardiac stomach	- 5.3	+ 5.9	-0.6
Pyloric stomach	+ 3.0	+ 0.7	-1.2
Small bowel	- 3.3	+ 0.6	0.0
Caecum	- 8.0	- 0.7	-1.7
Colon	- 8.3	+ 3.4	-1.8
Heart	- 2.7	+ 1.8	-0.9
Kidneys	- 2.8	- 3.3	-1.3
Liver	+16.6	- 0.7	-0.5
Lungs	-16.7	- 2.3	-2.6
Muscle (ventral abdominal wall)	- 5.4	- 0.7	-6.2
Salivary (submaxillary) glands	- 3.8	+ 1.3	-4.6
Skin	-10.7	- 2.8	-2.2
Spleen	+ 2.8	- 7.1	-3.0
Testes	- 6.5	+ 2.4	-2.7
Thymus gland	- 5.3	+ 0.6	-7.6
Residual carcass	- 6.1	- 0.2	-5.9

1) Water content was measured as gram water per 100 grams dry weight of tissue. The results are expressed as mean percent change from controls, specifically as $(\bar{X}_d - \bar{X}_c) / \bar{X}_c \times 100$ where \bar{X}_d is the mean in the diazinon-treated rats and \bar{X}_c the corresponding mean in the controls. One asterisk indicates that $\bar{X}_d - \bar{X}_c$ was significant at $P = 0.05$ to 0.02 and two asterisks at $P = 0.01$ or less.

mortality rate of 14 %. The LD₅₀ \pm S. E. of diazinon was 415 ± 39 mg/kg which is insignificantly different from that in animals of group (a) fed laboratory chow. The estimated value of the maximal LD₀ was 251 mg/kg and of the minimal LD₁₀₀ 578 mg/kg. The mean \pm S. D. hours to death was 29 ± 13 death occurring earlier the higher the dose. The immediate cause of death was respiratory failure in deep hypothermic coma.

The clinical signs during the first day were similar to those of rats in group (a). Measurements at 24 hours are summarized in table 1. An oligodipsia and an oliguria occurred which were not seen in rats of group (a), and in addition, an aciduria rather than an alkalinuria. The

aciduria may be related to the fact that control rats fed Protein Test Diet, Normal had a more alkaline urine than the controls fed laboratory chow. It will be noted that the results in table 1 are reported as percent changes from respective controls. The urinary pH of diazinon treated rats in group (b) was actually insignificantly different from that in group (a) but as compared to controls given no diazinon, a difference was found as indicated in table 1. One or two other signs, such as hypothermia and haematuria, were more marked in group (b) than in group (a). Otherwise the signs of toxicity in group (b) were identical to that in group (a). Gross and microscopic observations at autopsy were identical in the two groups. Recovery of survivors was rapid with a few significant differences as compared with survivors of group (a) shown in table 1.

Group (c) Fed Protein Test Diet Low There were 23 deaths due to diet only, a mortality rate of 21 %. At 28 days, the animals weighed 57 ± 6 g. The $LD_{50} \pm S.E.$ of diazinon was 215 ± 26 mg/kg which is lower than that in rats of groups (a) and (b) at $P < 0.001$. The estimated maximal LD_0 was 52 mg/kg and the minimal LD_{100} was 378 mg/kg. The mean \pm S.D. hours to death was 17 ± 12 which was significantly less than that in group (b) and group (a). The clinical signs during the first day were similar to those in rats of groups (a) and (b). Clinical measurements at 24 hours are summarized in table 1. The only measurement significantly different from that of rats in group (b) was a somewhat greater degree of aciduria. At autopsy there were certain microscopic findings, shown in table 2, which were characteristic of the kwashiorkoric state rather than typical of diazinon intoxication. Survivors (table 1) had significantly less glycosuria and significantly more proteinuria than the rats of group (b) but otherwise did not differ from survivors in group (b).

Diazinon - Older Stabilization The preparation termed "Diazinon Unstabilized - Stabilized in Ardsley (FL 8722)" was given orally to 54 overnight-starved rats of group (a) in doses of from 50 to 500 mg/kg, each dose to 6 rats, as a pilot test in order to determine the $LD_{50} \pm S.E.$ This was found to be 271 ± 61 mg/kg. The clinical signs of toxicity were similar to those in rats given diazinon, stabilized by the new process and termed "Diazinon Technical, 91 4 / diazinon, ARS No. 235/68, Batch No. FL-6199".

Discussion

The acute oral LD_{50} of technical diazinon in rats was found to be 100 to 150 mg/kg by BRUCE *et al.* (1955) who note that a previous value of 235 mm³/kg, or about 260 mg/kg, had been reported. BRUCE *et al.* (1955) gave technical diazinon ("85 / active compound") dissolved in corn oil when they used diazinon as a "25% wettable powder" and gave it as a

5 / suspension in methylcellulose, the LD₅₀, expressed as active diazinon, was 265 mg/kg. GAINES (1960) found that the LD₅₀ of a sample of diazinon technical obtained in 1953 decreased on storage over a subsequent period of 6 years. SPENCER (1968) lists the oral LD₅₀ of "the technical product" as from 150 to 220 mg/kg in rats, his values apparently referring to results reported some years previously. Information provided by the manufacturer of diazinon revealed that a substance termed Epoxol 7-4 was added to Diazinon 50W some time after its manufacture in the early 1960s and at present this is added at the time of manufacture. The stabilizer is added to prevent any conversion of diazinon to the more toxic monothio tetraethyl pyrophosphate and the addition of the stabilizer immediately after manufacture is considered a more effective method of stabilization. Our results confirm that this is true in so far as the material stabilized immediately after manufacture had a higher LD₅₀ (466 ± 87 mg/kg) than the older product stabilized some time after manufacture (271 ± 61 mg/kg).

These various results indicate that the oral LD₅₀ of diazinon may vary with the method of manufacture, type of vehicle used to dissolve or suspend the pesticide, and possibly with age of the product. The experiments described in this report were done over a period of 6 months and in the following order of studies i.e. first on group (a) then studies on group (c) and finally studies on group (b). The fact that the LD₅₀ in group (b) was similar to that in group (a) suggests that little or no change in the chemical nature of diazinon had occurred during this study.

The clinical signs of diazinon intoxication were similar to the muscarinic, nicotinic and central nervous-system effects previously described by BRUCE *et al* (1955) and reviewed by DURHAM & HAYES (1962). Of the pathological signs found at autopsy BRUCE *et al* (1955) had previously reported hemorrhage of the kidneys and lungs and a gastroenteritis. The marked loss of weight found in the caecum may be related to the finding, cited in FAO/WHO REPORT (1965), that the caecum plays a role in the metabolism of diazinon.

BOYD & DE CASTRO (1968b) have reviewed data on the acute oral toxicity of pesticides given to albino rats previously fed kwashiorkorigenic diets. Based on a comparison of the LD₅₀ in rats of group (c) and group (b) a protein-deficient diet augmented the toxicity of captan by 26 times. Captan is a pesticide structurally related to thalidomide. On the same basis, endosulfan was 4 times more toxic, lindane, malathion and diazinon each about twice as toxic and carbaryl and dicophane slightly more toxic. The remarkable feature is not that the pesticides were more toxic in the kwashiorkoric rat but that they were not as toxic as one might have expected with the exception of captan and perhaps endosulfan. The animals of group (c) failed to grow during the 28 days on protein-deficient diet, appeared weak and cachectic, showed signs typical of kwashiorkor

and many of them died. PETERS & BOYD (1966) noted that cachectic rats need not necessarily be more susceptible to the toxic effect of drugs. They found that rats which developed cachexia when fed a rancid diet had relatively large livers and could withstand large doses of pentobarbital. After 28 days on a diet, the livers of rats in group (c) had the same percentage of body weight as those of groups (a) and (b) as noted by DE CASTRO & BOYD (1968). It would appear that the mechanisms of detoxication of most pesticides were not markedly inhibited by a low protein diet. If the same mechanisms apply to detoxication in man, it would appear that most pesticides could be used in countries using a low protein diet without the danger of a markedly augmented susceptibility to pesticide toxicity.

Summary

The acute oral $LD_{50} \pm S.E.$ of technical diazinon stabilized immediately after manufacture was 466 ± 87 mg/kg body weight of young male albino rats previously fed laboratory chow. The corresponding value in rats fed for 28 days after weaning on a purified diet containing 3.5% protein in the form of casein was 215 ± 26 mg/kg. When the protein content of the purified diet was increased to normal limits, the LD_{50} was the same as in rats fed laboratory chow. The clinicopathological syndrome of toxicity to diazinon was essentially the same in rats fed all three types of diet except for the addition of kwashiorkoric signs due to protein deficiency in rats fed the low protein diet. The immediate toxic signs were diarrhoea, salivorrhoea, dacryorrhoea, epistaxis, exophthalmos, tachypnoea, tremors, ataxia, listlessness, anorexia, diuresis, glycosuria, alkalinuria, and hypothermia. Death usually occurred at $\frac{1}{2}$ to 2 days following respiratory failure in deep hypothermic coma. At autopsy an acute gastroenteritis, a stress reaction, widespread dehydration and loss of weight in body organs, as well as extensive capillary venous vasodilation were found. Survivors rapidly recovered from the toxic effects of the pesticide.

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Determination of the Anticonvulsant Potency of Unmetabolized Trimethadione

By

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BUTLER and his colleagues (1952 - 1954) have shown that trimethadione (3,5,5-trimethyl-2,4-dioxooxazolidine) is demethylated to 5,5-dimethyl-2,4-dioxooxazolidine (dimethadione) in the organism of the rat, dog and man. Since the process is almost quantitative, and since the renal excretion of dimethadione is extremely slow the authors have suggested that the metabolite plays a considerable role in the anticonvulsant effect of trimethadione. This view was later confirmed by the clinical studies of JENSEN (1962) and CHAMBERLIN *et al.* (1965).

It thus seemed of interest to elucidate the anticonvulsant activity of unmetabolized trimethadione. This, however, was difficult in that there is no sensitive method for the quantitative determination of this drug in biological material, whereas dimethadione can easily be determined by U V -spectrophotometry (BUTLER 1953). The anticonvulsant effects of trimethadione and dimethadione were compared by TAYLOR *et al.* (1956) and by WITKOW *et al.* (1968). While the former of these authors only gave a brief report without any quantitative data, the latter investigators based their conclusions mainly on results obtained in the maximal electroshock test in which trimethadione is clearly less effective than against convulsions elicited by pentetrazole (FREY 1964), so that an exact value for the potency ratio between both drugs was not obtained.

It was therefore decided to estimate the anticonvulsant activity of unmetabolized trimethadione by comparing the intravenous ED₅₀s of both trimethadione and dimethadione by the pentetrazole seizure threshold test and concurrent determination of the dimethadione concentrations in the serum. Such an approach seemed to be more conclusive than

an attempt to inhibit demethylation by the known inhibitors of microsomal liver enzymes, since these compounds might very well interfere with the anticonvulsant activity of the drug investigated

Material and Methods

Experiments were done in mice of both sexes of the HAM/ICR strain weighing 22-27 g

For the determination of the ED50 in the pentetrazole seizure threshold test (SWENSSON *et al.* 1952), trimethadione and dimethadione were injected intravenously into the tail vein in a volume of 10 ml/kg. Pentetrazole was injected subcutaneously in a dose of 100 mg/kg either simultaneously with the anticonvulsants or after 30 min., 1 hour, 2 and 4 hours respectively. Animals not showing clonic seizures with loss of righting reflexes within 30 min. of the pentetrazole injection were considered protected. Groups of 10 mice were used for each dose and the ED50 were determined by the method of LITCHFIELD & WILCOX (1949). In the graphical presentation of fig. 2 the ED50 values are put into the middle of the observation period, i.e. at 15 min., 45 min., 75 min. and so on.

In parallel experiments mice received the ED50's thus determined and were bled by decapitation at the corresponding times. After pooling the blood of 4 mice the amount of dimethadione in the serum was determined by the method of BUTLER (1953). Six determinations were done for each time.

In a further series of experiments the time course of serum concentrations of dimethadione was followed after the intravenous injection of 300 mg/kg trimethadione and the equimolar dose of 270 mg/kg dimethadione, respectively. Again, the blood of 4 mice was pooled and 6 determinations were done for each time.

Both trimethadione and dimethadione were kindly supplied by Abbott Laboratories.

Results and Discussion

The time course of serum concentrations of dimethadione after the intravenous injection of an equimolar dose of trimethadione (300 mg/kg) and dimethadione (270 mg/kg) is shown in fig. 1. After the injection of dimethadione, the serum concentrations fall slowly with a half-life of about 27 hours. After the injection of trimethadione a steep rise in dimethadione concentrations is seen, the values after dimethadione being reached at 6 hours after which both curves run close together. This points to a quantitative demethylation of trimethadione which is in good agreement with the results of BUTLER (1953) in other species. Consequently all the anticonvulsant activity of trimethadione observed later than 4-6 hours after the administration of the drug must be considered to be due to the metabolite.

Table I gives the ED50 values determined at the respective times after the intravenous injection for both trimethadione and the metabolite as well as the serum concentration of dimethadione found with the ED50 at the respective times

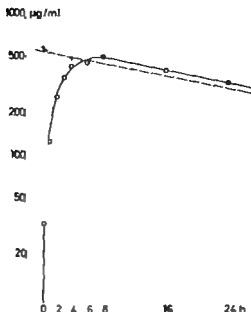


Fig. 1 Course of serum concentrations of dimethadione in mice injected intravenously with 270 mg/kg dimethadione (+ — +) or the equimolar dose of 300 mg/kg trimethadione (o—o).

In fig. 2 the log molar ED₅₀s for both compounds are plotted against time. All five values for trimethadione lie on a straight line and this is also true for dimethadione up to an interval of 1 hr 15 min. Both lines run parallel. By extrapolation to zero time, at which all the trimethadione is in the unchanged form zero time ED₅₀s of 2.2 mM/kg for trimethadione and 2.7 mM/kg for dimethadione are found, indicating that trimethadione has 1.21 times the anticonvulsant activity of its metabolite. With regard to the 2nd ED₅₀ for dimethadione, which deviates markedly from the straight line and results in much higher serum concentrations than the ED₅₀s for shorter time intervals, it must be remembered that the doses necessary for the construction of this dose effect curve all lie in the toxic range. Thus the highest dose of 1 g/kg had to be injected in two fractions with a 5 min. interval, and the ED₅₀ of 790 mg/kg had to be injected very slowly (in about 1 min.) in order to avoid deaths. Hence, the deviation at this point is thought to be the consequence of interfering unspecific and toxic actions.

The results offer a further possibility of calculating the anticonvulsant potency of unchanged trimethadione in relation to that of the metabolite, at least for the ED₅₀s determined for the time periods 0–30, 30–60 and

Table 1

Intravenous ED50's for trimethadione and dimethadione by the pentetrazole seizure threshold test and the dimethadione serum concentrations obtained with the corresponding ED50

		0-30 min. (15)	30-60 min. (45)	60-90 min. (75)	120-150 min. (135)	240-270 min. (255)
Trimethadione	ED50	335	350	390	480	660
	mg/kg	(300-370)	(320-390)	(340-440)	(420-540)	(560-770)
	[mM/kg]	[2.34]	[2.44]	[2.72]	[3.35]	[4.61]
	serum concentration DMO ¹⁾	33 ± 2.90	109 ± 13	178 ± 8.2	333 ± 43	632 ± 68
Dimethadione	ED50	360	400	430	790	
	mg/kg	(330-400)	(330-480)	(330-570)	(620-1000)	
	[mM/kg]	[2.78]	[3.1]	[3.33]	[6.31]	
	serum concentration DMO ¹⁾	891 ± 20	760 ± 18	856 ± 9	1360 ± 14	
	μg/ml					

¹⁾ dimethadione. ²⁾ B D

60-90 min. BUTLER (1953) has estimated a volume of distribution of 40% of body weight for dimethadione, a value which was confirmed in our experiments in mice. With the aid of this value and the dimethadione concentrations determined for the respective ED50's of trimethadione (table 1) it is possible to calculate the percentage that has been demethylated at the given time. The unchanged part of trimethadione should then be

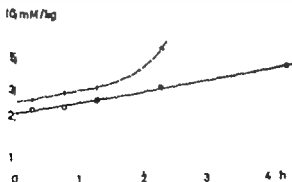


Fig. 2. Extrapolation of zero time ED50 for trimethadione and dimethadione from the ED50 values compiled in table 1

Table 2

Calculation of the relative anticonvulsant potency of unsaturated trimethadione. At each time the unchanged aliquot of the ED50 of trimethadione will be equieffective to the ED50 of dimethadione minus the aliquot of the ED50 of trimethadione demethylated to dimethadione (A in column (2)). The values in column (2) have been calculated from table 1 using volume of distribution of 40% of the body weight for dimethadione.

Time (1)	Trimethadione		Dimethadione ED50 - [A] (4)	Relative potency if trimethadione dimethadione = 1	
	demethylated (2)	unchanged (3)		molar	weight
0 min.	-	315 mg/kg = 2.2 mM/kg	345 mg/kg = 2.67 mM/kg	1.21	1.095
0-30 min.	4.4% = 14.7 mg/kg TMO ¹⁾ ~ 13.4 mg/kg DMO ²⁾ [A]	300 mg/kg	347 mg/kg	1.2	1.084
30-60 min.	13.7% = 48 mg/kg TMO ~ 44 mg/kg DMO [A]	224 mM/kg 302 mg/kg = 2.11 mM/kg	2.69 mM/kg 356 mg/kg = 2.76 mM/kg	1.31	1.18
60-90 min.	20% = 78 mg/kg TMO ~ 71 mg/kg DMO [A]	312 mg/kg = 2.18 mM/kg	359 mg/kg = 2.78 mM/kg	1.28	1.15

1) trimethadione.

2) dimethadione.

equieffective to the corresponding ED₅₀ of dimethadione minus the amount that had already been demethylated in the experiments with trimethadione (table 2). The relative potency thus calculated for four different points of time shows satisfactory agreement and allows of the conclusion that on a molar basis the unmetabolized trimethadione has 1.25 ± 0.05 times the anticonvulsant potency of dimethadione. On a weight basis the potency ratio is 1.13 ± 0.05 .

Summary

In mice the intravenous ED₅₀'s of trimethadione and its metabolite dimethadione have been determined by the pentetrazole seizure threshold test at different time intervals after the injection. Concurrently the serum concentrations of dimethadione were determined. The results obtained in these experiments show that the anticonvulsant potency of unmetabolized trimethadione on a molar basis is 1.25 times that of dimethadione.

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Metabolism, Distribution and Excretion of Flupenthixol

By

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Flupenthixol (fluanxol®) (4-(3-(2-trifluormethylthioxanthen-9-ylidene)propyl)-1-piperazine-ethanol) was synthesized as a member of a series of thioxanthen derivatives (PETERSEN & MØLLER-NIELSEN 1964) and selected for clinical trial on the basis of its pharmacological properties (MØLLER-NIELSEN 1967). The present publication deals with the biotransformation of flupenthixol in dogs and rats and the quantitative aspects of the distribution and excretion of the compound in rats.

Materials and Methods

A. Labelled compound.

Flupenthixol was labelled by the Witzsch method as described by BRUNSTRÖM & LUNDSTRÖM (1957). About 500 mg of the finely powdered HCl-salt was exposed to ^1C tritium-gas for 3-4 weeks. The substance was purified by recrystallization and precipitated as HCl-salt. The radiochemical purity was found to be $>90\%$ by thin-layer chromatography.

The specific activities of the compound used were

Distribution study: 8.9 $\mu\text{Ci}/\text{mg}$.

Excretion study on oral administration: 10.6 $\mu\text{Ci}/\text{mg}$.

Excretion study on intravenous administration: 9.0 $\mu\text{Ci}/\text{mg}$.

Bile collection study: 9.0 $\mu\text{Ci}/\text{mg}$.

B. Animal studies.

Biotransformation studies. Male Wistar rats were given a single dose of 25 mg/kg of unlabelled flupenthixol by intraperitoneal injection and the urine and faeces collected for the following 24 hours.

Two beagle dogs were given daily doses of 5 mg/kg of unlabelled flupenthixol by intramuscular injection for four days and the urine was collected during this period. All urine samples were pooled before analysis.

Distribution study Male Wistar rats (90–110 g) fasted for 16 hours were given a single oral dose of 10 mg/kg of labelled flupenthixol in distilled water (10 ml/kg). Groups of three rats were then sacrificed by exsanguination under ether anaesthesia 30 minutes, 1, 2, 4 and 8 hours, and 1, 2, 3, 6, and 10 days after administration of the drug. Blood samples were collected in tubes containing EDTA to prevent clotting. The brain, lungs, liver, heart, kidneys, spleen, epididymal fat, gastro-intestinal tract and carcass were examined.

Excretion studies 10 male Wistar rats (90–110 g) fasted for 16 hours were given 10 mg/kg of labelled flupenthixol orally or intravenously in distilled water (10 ml/kg). The urine and faeces were collected separately. In the study with oral administration, the collection times were 1, 2, 4, 8, and 24 hours and thereafter each day until ten days after administration. In the investigation with intravenous injection the times of collection were 1, 4, 8, 24, 48, 72, 96, and 120 hours.

Bile collection study Four male Wistar rats (210–230 g) fasted for 16 hours were anaesthetized with urethane (1.5 g/kg i.p.). A polythene drain was inserted into the common bile duct and the bile was collected. The bile collected for the first hour served as control bile. The rats were then given 2 mg/kg of labelled flupenthixol intravenously and the bile was collected for five hours.

C. *Assay technique*

Samples were measured in a Beckman Liquid Scintillation Counter with automatic external standardization. With the exception of the blood samples, the scintillation medium used was 10 ml of the dioxane-methanol-toluene-naphthalene mixture (diotol) as described by HAMANO (1960), modified by replacing POPOP with dimethyl-POPOP. With the blood samples toluene/triton X 100 (2/1) medium was used containing 6 g/l PPO and 0.3 g/l dimethyl-POPOP.

D. *Extraction*

In the studies with the unlabelled compound, extractions were made on the urine and faeces of rats and on the urine of dogs at basic and acidic pH. Extraction was performed with three portions of ether or ethylene dichloride (EDC) at each pH. The organic phases were dried over anhydrous sodium sulphate and evaporated to dryness at low temperature in vacuum. The remaining material was redissolved in 1–2 ml EDC or ethanol and used for chromatography. Acid or β -glucuronidase hydrolysis was performed on urine before or following basic extraction.

In the studies with labelled compound urine as well as faeces- and tissue homogenates were extracted 3–4 times with EDC at pH 9. The combined extracts were concentrated by evaporation in vacuum, an aliquot was taken for counting of radioactivity and another one used for chromatography. Acid- or β -glucuronidase hydrolysis was made following extraction and another extraction made as described above.

E. *Chromatography*

Thin-layer chromatography (TLC) was performed on glassplates (20 x 20 cm) coated with a .50 μ layer of Silica Gel G according to STANL (Merck) and activated at 110°C for 30 minutes.

The following solvent systems were used

1. Benzene diethylamine dimethylformamide 80/10/10
(unsaturated)

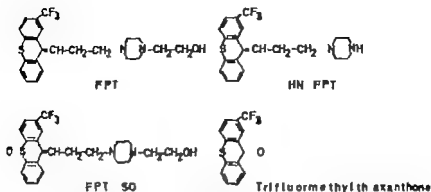


Fig. 1. Reference substances. Flupenthixol (FPT), flupenthixol-sulphoxide (FPT-SO), 2-trifluormethyl-3-(3-N-piperazinyl-propyliden)-thioxanthone (HN FPT) and 2-trifluoromethyl-thioxanthone.

2. Acetone: heptane: diethylamine (saturated)	60 40 10
3. Ether: diethylamine (saturated)	90 10
4. Butanol: acetic acid: water (saturated)	80 20 20
5. Isopropanol: benzene: 1% ammonia (unsaturated) (upper phase was used).	35 45 20

For the detection of spots the following spray reagents were used

Sulphuric acid, conc. (UV light) (all compounds)

Perchloric acid (sulfoxides) (SMITH 1960)

5% FeCl₃-solution (phenols) (STAM 1967)

Fast Blue Salt II (phenols) (STAM 1967)

Iodoplatinate reagent (N-compounds) (SMITH 1960)

Flupenthixol (FPT), flupenthixol-sulphoxide (FPT-SO), 2-trifluormethyl-3-(3-N-piperazinyl-propyliden)-thioxanthone (HN-FPT) and 2-trifluormethyl-thioxanthone were used as reference substances (for formulas see fig. 1).

FPT and HN-FPT were exposed to gentle oxidation by heating an 0.5% ethanolic solution adjusted to pH 4 with one tenth the volume of 30% H₂O₂-solution over night in an oven at 40. The solution was chromatographed directly.

After chromatography of radioactive extracts the silica gel was scraped off into a separate counting vial for each centimeter beginning at the starting point, and the radioactivity determined.

UV absorption curves.

UV absorption curves of reference substances were made in 0.1 N HCl-solutions. Absorption curves of spots on the chromatogram were made by scraping off the spot containing area, eluting it with 0.1 N HCl-solution and filtering through glass filter G 4. A blank made from the plate was used for correction. The absorption curves were read in a Zeiss UV PMQ spectrophotometer against 0.1 N-HCl-solution.

Table I

 R_f -values of M_0 , M_I , M_{II} , M_{III} and reference substances.

	System 1	System 2	System 3	System 4	System 5
M_0	0.85	0.57	0.27	0.37 0.33	0.60
M_I	0.77	0.45	0.14	0.17	0.39
M_{II}	0.51	0.30		0.45	
	0.41	0.25			
M_{III}	0.41	0.20	0.06	0.29	0.07
	0.32	0.15			
FPT	0.84	0.58	0.29	0.37 0.33	0.60
FPT-SO	0.74	0.45	0.15	0.17	0.34
HN FPT	0.52	0.30	0.11	0.45	0.22
	0.42	0.23			
FPT + H_2O_2	0.74	0.45		0.17	0.34
HN FPT + H_2O_2	0.41	0.20		0.29	0.07
	0.32	0.15			
Trifluoromethyl-thioxanthone	0.98	0.86			

Results

Biotransformation of flupenthixol

Chromatography of urine extracts indicated the presence of four compounds, named M_0 , M_I , M_{II} , and M_{III} . R_f -values are given in table I. M_0 , M_I and M_{II} had R_f -values and gave colour reactions with the spray reagents identical to authentic FPT, FPT-SO and HN FPT respectively. The U V absorption curve of M_I was shown to be identical to the U V absorption curve of authentic FPT-SO (fig. 2), with a shoulder at about 250 m μ and a maximum at 216 m μ . Authentic FPT and HN FPT had maxima at 204 m μ and 228 m μ and a shoulder at about 265 m μ . The U V absorption curve of M_{III} (fig. 3) showed the same characteristics as the curve of M_I and authentic FPT-SO, indicating that M_{III} might be a sulphoxide. In addition it gave orange fluorescence in U V light after spraying with conc. sulphuric acid and green fluorescence after spraying with persulphate reagent as did authentic FPT-SO while FPT and HN FPT gave a yellow fluorescence with the two spray reagents. Oxidation by H_2O_2 of FPT and HN FPT gave, in addition to traces of the parent compounds, spots on the chromatograms which had R_f -values identical to authentic FPT-SO and M_{III} respectively. This formation of M_{III} from

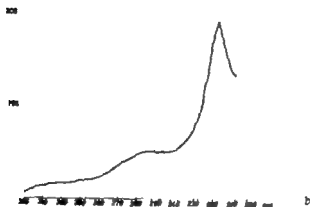
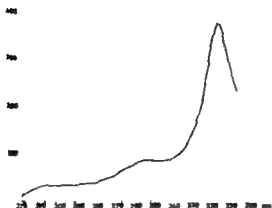


Fig. 2. UV absorption curves of authentic FPT-SD (a) and M (b).

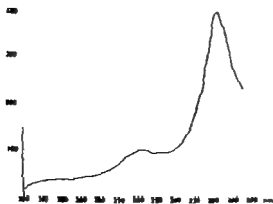


Fig. 3. UV absorption curve of Mnt.

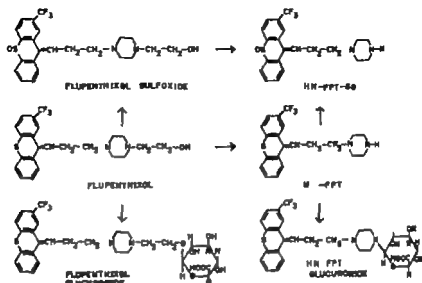


Fig. 4 Biotransformation of flupenthixol.

HN-FPT in addition to the UV absorption characteristics and colour reactions of M_{III} , makes it reasonable to assume that the metabolite M_{III} is identical with HN-FPT-SO.

Thus the four excretion products are considered to be FPT, FPT-SO, HN-FPT and HN-FPT-SO indicating the biotransformation shown in fig. 4. In all chromatograms a fifth spot corresponding to trifluormethyl-thioxanthone (for formula see fig. 1) is seen, but this substance is most likely not a metabolite, as it is always formed in small quantities by chemical manipulations with the thioxanthenes.

In urine from rats, but not from dogs more FPT and HN-FPT are extractable after acid or β -glucuronidase hydrolysis, indicating that these two compounds are to some extent bound as glucuronides in urine from rats. No phenolic metabolites could be detected.

Urine from rats and dogs contains mainly the two sulphoxides and only small amounts of FPT and HN-FPT in the free form. In the faeces only FPT and small quantities of HN-FPT could be detected.

Distribution study

The distribution of radioactivity following oral administration of 3H labelled flupenthixol is shown in table 2 and fig. 5. The blood concentrations are shown in table 3.

Apart from the gastro-intestinal tract, which contained unabsorbed as

Table 2

Content of radioactivity in tissues expressed as $\mu\text{g flupenthixol/g wet weight}$ and % of dose. (— 3).

	1 hour		4 hours		8 hours		1 day		6 d. ys	
	$\mu\text{g/g}$	%-dose	$\mu\text{g/g}$	%-dose	$\mu\text{g/g}$	%-dose	$\mu\text{g/g}$	%-dose	$\mu\text{g/g}$	%-dose
Brain	0.8	0.1	2.2	0.3	1.7	0.3	0.8	0.1	0.3	0.0
Heart	4.4	0.2	5.2	0.2	4.6	0.2	2.8	0.1	0.8	0.0
Lungs	18.7	1.6	33.5	2.7	39.1	2.3	6.6	0.3	0.6	0.0
Liver	30.2	12.3	31.2	11.4	24.8	9.3	10.0	4.6	1.4	0.1
Kidneys	9.9	0.9	12.5	1.1	10.2	0.9	3.1	0.4	0.8	0.1
Spleen	8.1	0.4	13.4	0.6	12.7	0.5	6.4	0.2	0.7	0.0
Adipose	2.2	0.1	3.4	0.2	3.1	0.2	3.0	3.0	0.4	0.0
Intestine	41.0	65.2	67.9	60.8	33.8	32.9	19.7	21.1	0.1	0.2
Whole body	4.1	10.6	3.1	20.8	3.5	22.8	1.7	11.2	0.1	2.1
at time		4.14		92.2		29.3		18.2		1.2

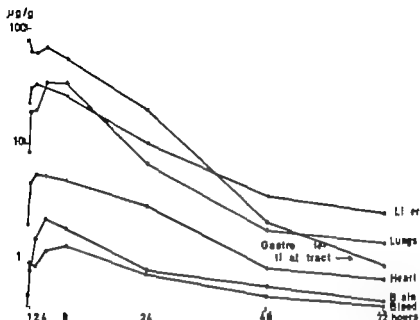


Fig. 5 Distribution of radioactivity following oral administration of ^3H -flupenthixol. Ordinate: Radioactivity expressed as μg flupenthixol/g wet weight.

well as excreted substance, the greatest concentrations were seen in the liver and the lungs. The lowest concentrations were found in the brain and the blood. Ten days after administration radioactivity could be determined only in the liver, kidneys and spleen.

Extraction and chromatography were performed on pooled organs. The organ, time after administration and per cent of total radioactivity extractable at pH 9 are shown in table 4. It is seen that in the brain, the extractable part of radioactivity decreased with time. Acid hydrolysis of brain homogenates after the first extraction did not release more radioactivity for extraction, indicating that the remaining radioactivity was strongly bound. Chromatography of the extracts showed that the brain and the liver contained almost exclusively the unchanged FPT with only traces of metabolites, while the lungs as well as the parent compound contained a small quantity of FPT-SO.

Excretion studies

a. Oral administration The cumulative excretion of radioactivity in the urine and faeces following oral administration of ^3H -flupenthixol is shown in fig. 6. Total excretion of radioactivity was greatest in the faeces, in which more than 63% of the administered dose was found. The urinary

Table 3

Blood concentrations expressed as
 μg flupenthixol/ml ($n = 3$).

	$\mu\text{g/ml} \pm \text{S.D.}$
30 min.	0.37 ± 0.03
1 hour	0.90 ± 0.04
2 hours	0.84 ± 0.13
4 hours	1.14 ± 0.01
8 hours	1.24 ± 0.17
1 day	0.71 ± 0.06
2 days	0.46 ± 0.09
3 days	0.38 ± 0.00
6 days	0.26 ± 0.04
10 days	0.10 ± 0.02

excretion accounted for 17.4% of the dose. 2.2% remained in the body at the end of the ten days period. Excretion in the urine was greatest between 4 and 8 hours (about 1% of the dose/hour). Excretion in the faeces was greatest in the period 8 to 24 hours being about 1.6%/hr.

About fifty per cent of the radioactivity present in urine could be extracted without hydrolysis. Hydrolysis using β -glucuronidase or hydrochloric acid made another 5-11% of the radioactivity extractable.

Table 4

Per cent radioactivity extractable
 at pH 9

	Time after administration hrs	% extractable radioactivity
Brain	2	91.6
	4	86.1
	8	74.5
	24	37.2
Lungs	4	91.8
	8	90.4
Liver	2	87.1
	4	87.4

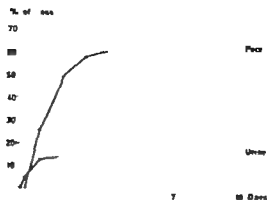


Fig. 6. Cumulative excretion of radioactivity following oral administration of ^3H -flupenthixol.

Chromatography of the extracts showed that the main excretion product in urine was FPT-SO. Smaller quantities of FPT and HN-FPT-SO were found. After hydrolysis using β -glucuronidase or hydrochloric acid more FPT and HN-FPT could be detected indicating a conjugation of these two compounds with glucuronic acid.

About 54% of the radioactivity present could be extracted in the faeces obtained between 8 and 24 hours. From faeces obtained at later periods relatively small quantities could be extracted. Acid and β -glucuronidase hydrolysis gave slightly more extractable radioactivity but in the latter case, it was not a specific effect of the β -glucuronidase, since equal quantities could be extracted from a control sample treated in the same way without the addition of β -glucuronidase. Many impurities extracted from faeces interfered with the chromatographic analysis. The main

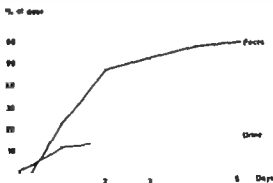


Fig. 7. Cumulative excretion of radioactivity following intravenous injection of ^3H -flupenthixol.

excretion product in the faeces was unchanged FPT. Small quantities of HN-FPT were also seen.

b *Intravenous administration.* The cumulative excretion of radioactivity following intravenous injection of ^3H flupenthixol is shown in fig. 7. The total excretion in the faeces and urine was 59.3% and 16.4% of the dose respectively. 14.4% remained in the body at the end of the five days period.

The excretion patterns following oral and intravenous administration were very similar. The only difference seen was in the urinary excretion during the period 0-4 hours. This difference is most likely due to a more rapid distribution after intravenous injection than after oral administration.

In the oral and intravenous excretion studies 83% and 90% respectively of the administered dose could be accounted for. It should be noted, however, that small quantities of radioactivity were expired as $^3\text{H}_2\text{O}$. This most likely originated from the radioactivity situated in the ethanolic group, which was oxidized to CO_2 and water after this group had been split off and HN flupenthixol had been formed. In a separate study the expired water collected for a short period of time did in fact contain some radioactivity. It is difficult, however, to make long lasting studies of this kind and in consequence it was not possible to obtain any quantitative data.

Bile collection study

Bile collection from four rats during the first five hours following an intravenous dose of 2 mg/kg flupenthixol yielded 6.5 ml of bile containing 12.6% of the dose given.

Discussion

The biotransformation pattern of flupenthixol suggested in fig. 4 indicates that sulfoxidation and dealkylation in the side chain are the main routes of degradation. This is in good agreement with that found for the other thioxanthenes, chlorprothixene (HUUS & KHAN 1967; RAAPLAUS 1967) and clopenthixol (KHAN 1968). It is remarkable that no phenolic metabolite could be detected, since other tricyclic psychotropic drugs e.g. chlorpromazine (HUANG 1967) and imipramine (CRAMER & SCOTT 1966) form phenolic metabolites in considerable amounts.

The distribution of flupenthixol does not differ substantially from that of other similar neuroleptic drugs e.g. fluphenazine (ESERT & HIRS 1965). It is remarkable that the lowest concentration seen in any organ was in

the brain. Peak concentrations in the brain and most other organs were seen, four hours after oral administration.

The distribution study indicated a biological half-life of about 16 hours. From the excretion studies, half-lives of about 22 hours and about 27 hours could be estimated, following oral and intravenous administration respectively. The substantial faecal excretion seen after oral administration might be thought to indicate incomplete absorption. However, since the same excretion pattern was seen following intravenous injection, this possibility is not likely. The large biliary excretion indicates a considerable enterohepatic circulation and the substance from this circulation, escaping reabsorption, could account for the excreted substance found in faeces. However, excretion by the salivary glands, the sudoriferous glands and the pancreas should also be considered. The nature of the non-extractable radioactivity cannot be accounted for, but this radioactivity seems to be bound by a strong and rather unspecific binding, as this phenomenon is also described for other substances (EBERT & HESS 1965; G. PLYM-FORSHELL *et al.*).

The excretion pattern showing excretion via the faeces to be 4-5 times the urinary excretion is in good agreement with results published on phenothiazines with a piperazine side chain (SYMCHOWICZ *et al.* 1962; FLANAGAN *et al.* 1962). The major faecal excretion seen with these compounds is possibly a consequence of the structure of the side chain, as the urinary excretion of chlorpromazine and chlorprothixene, which have no piperazine group in the side chain, is almost equal to the faecal excretion.

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Effect of Terodiline on Cardiac and Brain Catecholamines in the Rat

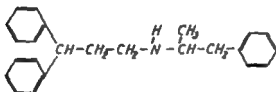
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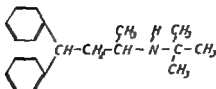
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It has been demonstrated that in rats prenylamine lowers the noradrenaline content of the heart to about 40% of its normal value when administered during seven days in a dose of 40 mg/kg/day (NIELSEN &

PRENYLAMINE
(SEGONTIN® SEDOLATAN®)



TERODILINE
(AKOR®)



OWMAN 1967). Since it has been claimed that this drug used in the treatment of anginal pain may act by interfering with the catecholamines of the heart (LINDNER 1961) it seemed justifiable to test the corresponding effect of another drug, terodiline, with the same indication as and structurally similar to prenylamine (fig. 1)

Material and Methods

Albino rats of both sexes weighing 200–250 g were used. Terodiline was administered subcutaneously to the experimental animals for doses see table 1. Animals given physiological saline served as controls. The animals were killed by decapitation after a blow on the neck after which the heart and brain were immediately dissected out, weighed and homogenized in perchloric acid at 0–4°. The catecholamines were determined by the procedure of BARTLER *et al.* (1958) as modified by HÄGGGREN *et al.* (1963).

The results are presented in table 1

Results and Discussion

When given 100 mg/kg/day or more about half of the animals died with seizures on the second or third day. All the animals which received 50 mg/kg/day for seven days survived. After two to four days, a clear sedation of the animals was observed. Thus spontaneous activity estimated subjectively decreased and the animals were easier to handle. Unfortunately this parameter was not assessed quantitatively. No significant changes in the total amount of catecholamine in the heart or brain

Table 1

Catecholic amine content of the rat heart and brain after administration of terodiline
CA = catecholic amine, NA = noradrenaline, DA = dopamine

Treatment of animals	No. of animals	Brain CA content		Heart CA cont.	test
		NA µg/g ± S.D.	DA µg/g ± S.D.	NA µg/g ± S.D.	
Terodiline, 100 mg/kg 24 hrs NaCl	6	0.41 ± 0.05	0.47 ± 0.08	1.23 ± 0.12	2.04 n.s. *)
0.9% 24 hrs Terodiline, 50 mg/kg/d 7 days NaCl	3	0.41 ± 0.03	0.49 ± 0.02	1.04 ± 0.11	
0.9% 7 days	III	0.45 ± 0.05	0.55 ± 0.11	1.40 ± 0.30	0.05 n.s.
	6	0.45 ± 0.04	0.57 ± 0.12	1.47 ± 0.18	

*) n.s. = not significant.

were found. This must, however not be interpreted as showing that catecholamines are of no significance in the subject studied. This finding may well be due to differences in the method used since only the total amount of the amines is estimated.

It is interesting to note that the two antianginal drugs discussed above have such different effects on the catecholamine content of the heart. Thus it must be questioned whether prenylamine produces its therapeutic effects by interfering with the catecholamine metabolism.

Another point of interest is that the amine-depleting effect of prenylamine might be confined to the amphetamine-part of the molecule. Thus if this part is replaced by a tertiary butyl group as in terodiline, no effect on the catecholamines can be detected. Moreover it has recently been shown, that in the rabbit and man prenylamine is metabolized to amphetamine and amphetamine metabolites (PALM & GROBECKER 1968) and it still remains to be elucidated whether prenylamine *per se* has a depleting effect on the catechol amines or whether this is caused by its metabolites.

Summary

A new drug, terodiline, used in the treatment of anginal pain has been investigated for its possible effect on the heart cardiac and brain catecholamines in the rat. In spite of its chemical similarity to prenylamine, another anti-anginal drug, known to be a potent releaser of catecholamines, no effect on catecholamine stores could be detected after the administration of terodiline.

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Erratum - Investigation on the Toxicity of Small Chronic Doses of Tannic Acid
with Special Reference to Possible Carcinogenicity *Acta pharmacol.*
et toxicol. 1968, 26, 41-45

The director of food research at Thomas J Lipton, Inc., Mr Harold N Graham has called our attention to an error in our paper entitled "Investigation on the Toxicity of Small Chronic Doses of Tannic Acid with Special Reference to Possible Carcinogenicity" which appeared in *Acta pharmacologica et toxicologica* 1968 26 41-45 and in which we stated that "the daily intake of tannic acid is generally rather high. 100-500 mg is found in a cup of tea, cocoa or coffee" We have defined tannic acid as a complex of esters of D-glucose with gallic and galloyl-gallic acids.

Mr Harold N Graham has informed us that neither tea, coffee nor cocoa contain significant amounts of the galloyl or digalloyl esters of glucose, that is to say they do not contain tannic acid as the term is usually understood, although, it is correct to say that there are "tannin-like" materials present.

We very much regret the wrong reference to a paper by B Korpassy *Cancer Research* 19 503 1959

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Effects of Felypressin (Octopressin®) on the Acute Toxicity of Local Anaesthetics

By

Bengt Åkerman

(Received April 28, 1969)

The synthetic polypeptide felypressin (octopressin ® from Sandoz Ltd., Basle, Switzerland) has been found to increase the activity of several local anaesthetics (HOCHULI 1962 MISSURA & WEDER 1962 DE GRENIS 1963 KLINGENSTRÖM, NYLÉN & WESTERMARK 1967). Though it resembles natural vasopressin in that it has a constrictor effect on small blood vessels, felypressin differs in exhibiting only a weak oxytocic action (BERDE, WEIDMANN & CERLETTI 1961). Recent studies have shown that the effectiveness of solutions of lidocaine and prilocaine are enhanced both in animals (ÅKERMAN 1966) and man (BERLING 1966 BROWN 1968) by concentrations of felypressin lower than those previously used.

Although the clinical tolerance following subcutaneous or e.g. epidural injection of the local anaesthetics in man is increased by vasoconstrictors of the catecholamine type, it has been difficult to demonstrate any decrease in the subcutaneous toxicity in animals (HOLLER 1952 AVANT & WEATHERBY 1960 ÅSTRÖM, PERSSON & ÖRTENGREN 1964). Since the mode of action of felypressin has been found to differ from that of the catecholamines (BERDE, SCHALCH & DOEPFNER 1964), it might be expected that the toxicity of local anaesthetic solutions is influenced by felypressin in another way than for example, in the case of adrenaline. The purpose of the present investigation was to elucidate the acute toxicity of solutions of local anaesthetics containing felypressin as a localizing agent and to compare the findings with the results obtained with solutions containing the most commonly used vasoconstrictor adrenaline.

Material and Methods

Albino mice (D3L, 17-23 g) were used. The study was carried out between 9 a.m. and 3 p.m. at a room temperature of 19-22°. The animals were kept under identical conditions before and during the experiments. The symptoms of central nervous toxicity after subcutaneous administration of varying sublethal doses of tetracaine, lidocaine, prilocaine and procaine given in a dose of 0.2 ml/20 g body weight were observed in a pilot experiment. The experiments were performed by two technicians, one of whom injected the substance into the backs of the animals, while the other measured the latent periods before the appearance of convulsions and loss of the righting reflex, as well as the duration of the toxic condition. The doses which produced convulsions of about the same duration were selected for a second series of experiments in which felypressin or adrenaline was added to the solutions. The concentration of felypressin varied from 0.01 to 1.0 IU/ml corresponding to 0.18-18.0 µg/ml of the synthetic polypeptide, and the adrenaline concentration from 1:400,000 to 1:100,000 (2.5 to 10.0 µg/ml). Each solution was tested in 10 animals. Owing to the considerable number of solutions, different batches of mice had to be used. However each particular agent was tested on a given batch. The results of control experiments showed small variations between the different test periods. The onset time of convulsions after three standard injections of 200 mg/kg of prilocaine for example, was found to be 3.6 ± 0.3 , 6.5 ± 0.8 , 7.0 ± 0.3 min. The corresponding durations were 26.4 ± 2.0 , 27.0 ± 0.9 and 29.0 ± 1.0 min.

The plasma concentrations of lidocaine (50 and 100 mg/kg) and prilocaine (100 and 200 mg/kg) were also determined in mice after subcutaneous injection of plain solutions (1.0 and 0%) and of solutions to which felypressin or adrenaline had been added. Each solution was injected into three groups of 10 animals. One group of mice was sacrificed 5 min. after the injection, the other two groups after 15 and 30 min., respectively. After centrifugation of the blood, 0.3 ml of the plasma from each mouse in the groups of 10 animals was pooled. The amounts of the local anaesthetics in the samples were determined by gas chromatographic analysis (SVENSSON, ÖRTENGREN & JACOBSON 1966).

Plain solutions of tetracaine (0.01%), lidocaine (0.05%), prilocaine (0.05%) and procaine (0.05%) as well as solutions containing felypressin or adrenaline were infused intravenously into mice of the same strain as above. The solutions were administered at two different rates (3.6 and 8.25 ml/hr) into the tail vein via a plastic catheter connected to a 10 ml syringe driven by a Braun constant rate infusion pump. The animals were not restrained except during the initial phase when they were fixed in a rubber tube in order to facilitate proper insertion of the needle.

The effects on the heart rate in mice were studied by the method of ROSS (1963). The heart rate of mice anaesthetized with pentobarbital sodium (80 mg intraperitoneally) was calculated from ECG records on Grass Model 5 Polygraph. The drugs were given in doses of 0.2 ml/20 g body weight.

The local anaesthetics were used as the hydrochlorides and adrenaline as the bitartrate. Addition of felypressin was made from stock solution (Mandox, Basle) of the following composition: synthetic PLV 1, 25 ± 3 IU (18 µg of PLV 2 corresponds to 1.0 IU) sodium acetate, 2.0 mg; glacial acetic acid, 1.0 mg; sodium chloride, 0.3 mg; trichlorobutyl alcohol, 2.0 mg and distilled water to 1.0 ml. The pH of the solution was 4.5. A solution containing only the preservatives was also used, the composition of which was the same as that mentioned above. Only fresh solutions prepared in 0.85% NaCl were used, the pH being adjusted to 6.8-7.0.

Results

Toxic doses of local anaesthetics generally produce successive stages of systemic intoxication such as tremors convulsions and loss of the righting reflex. When injected subcutaneously into mice, 25 mg/kg of tetracaine, 100 mg/kg of lidocaine and 200 mg/kg of procaine and prilocaline produced these symptoms in all the animals for about equal periods of time. Addition of felypressin and adrenaline to the solutions decreased the frequency of the convulsions and loss of the righting reflex (fig. 1). In those cases in which these symptoms did not disappear completely felypressin prolonged the onset time and shortened the duration of the toxic state (fig. 2). While 0.01 IU/ml had only a weak effect, significant protection was obtained by 0.05 IU/ml of felypressin in the solutions of prilocaline lidocaine and tetracaine. In procaine solutions the best effect was achieved by 0.2 IU/ml. The localizing properties decreased with in-

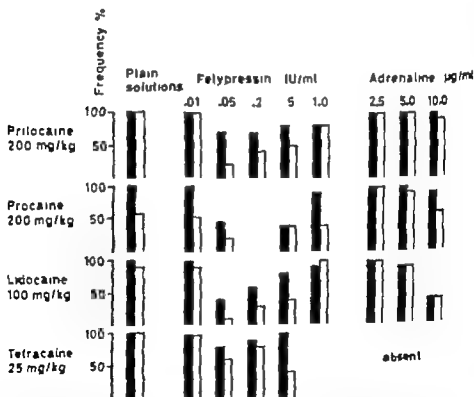


Fig. 1 Effects of felypressin and adrenaline on the frequency of convulsions (black bars) and loss of the righting reflex (open bars) following subcutaneous injection of local anaesthetics into mice. The gents were injected 0.20 ml/20 g body weight. Each solution was tested on 10 animals.

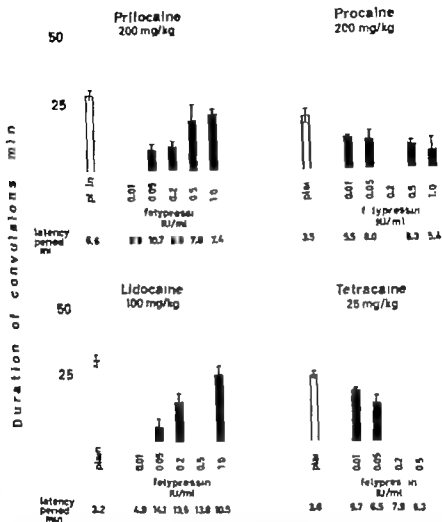


Fig. 2. The effect of felypressin on the duration of convulsions caused by local anaesthetics on subcutaneous injection of 0.20 ml/20 g body weight into groups of 10 mice per solution. Latent period before appearance of the toxic state indicated by numbers.

creasing concentration of felypressin. The effect of solutions of the local anaesthetics containing the preservatives (see Methods) but not the synthetic polypeptide did not differ from that of the plain solutions. As shown in figs. 1 and 3 tetracaine and lidocaine were influenced to a greater extent by adrenaline in the concentration range 2.5–10.0 $\mu\text{g/ml}$ than were procaine and prilocaine. With the latter three agents, small amounts of adrenaline prolonged the latent period to convulsions, but

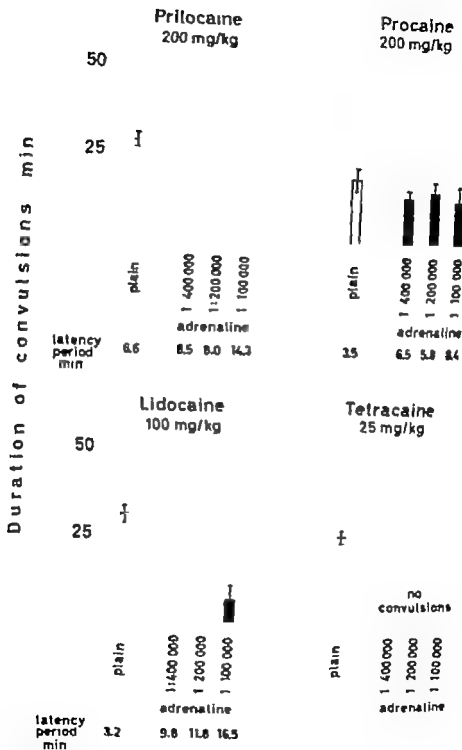


Fig. 3 Effect of adrenaline on duration of convulsions caused by local anesthetics. 0.20 ml/20 g was injected subcutaneously to groups of 10 mice. Onset time indicated by numbers.

only the highest concentration tested (1:100,000) had any marked effect on the frequency of the toxic symptoms and the convulsion time.

Felypressin effectively reduced the rate of absorption of the local anaesthetics as indicated by the plasma concentrations of prilocaline and lidocaine following subcutaneous injection into mice (table 1).

Apparently a concentration of 0.05 IU/ml was more active than 0.005 and 0.5 IU/ml. The effects of 0.05 IU/ml of felypressin and of 10.0 µg/ml of adrenaline were very similar in conjunction with the highest dose of prilocaline (200 mg/kg) felypressin reduced the plasma levels much better than adrenaline. The results obtained with the different concentrations of felypressin and the difference in effect between felypressin and adrenaline, depending on the local anaesthetic used are in good agreement with the findings of the previous series of experiments. It should be noted that the peak plasma levels in the absence of vasoconstrictor were higher for lidocaine than for prilocaline. The amounts in the plasma 15 min. after administration of 100 mg/kg of lidocaine and 200 mg/kg of prilocaline were almost the same (15.7 and 15.5 µg/ml, respectively).

The effects of felypressin and adrenaline on the intravenous toxicity of tetracaine, lidocaine, procaine and prilocaline in mice are shown in fig. 4. The lethal doses of the compounds were decreased by either of the two adjuncts at two different rates of infusion. However adrenaline

Table 1

Concentrations of lidocaine and prilocaline in plasma at different times after subcutaneous injection into mice. The amounts of the agents were determined from a pool to which 0.3 ml plasma from each of 10 mice was added.

Compound	Dose mg/kg	Time after injection min.	Amount local anaesthetic, µg/ml, base				
			Plain solutions	Felypressin, IU/ml			Adrenaline, µg/ml
				0.005	0.05	0.5	10.0
Lidocaine	50	5	9.8	5.2	3.9	4.7	2.7
		15	8.4	5.7	4.5	5.7	5.3
		30	3.2	3.4	3.1	3.3	3.6
	100	5	8.1	—	7.3	7.0	6.4
		15	15.7	—	9.9	13.2	9.4
		30	6.7	—	6.1	6.1	5.8
Prilocaline	100	5	10.2	—	3.9	5.2	3.6
		15	6.7	—	7.2	8.8	7.7
		30	4.6	—	5.2	4.5	5.9
	200	5	10.8	—	8.0	9.8	9.1
		15	15.5	—	10.5	14.4	18.1
		30	8.1	—	8.5	9.4	11.8

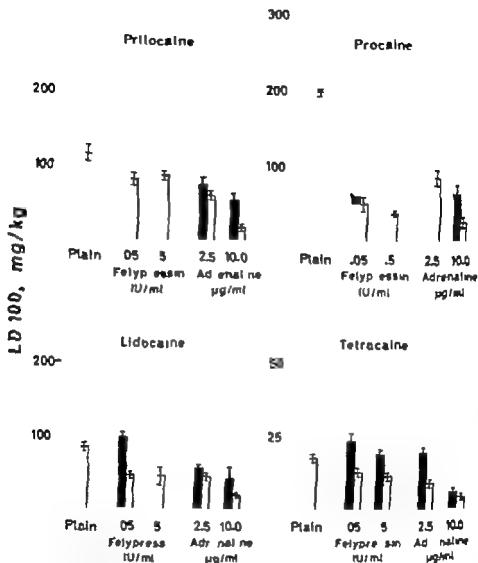


Fig. 4. Effects of felypressin and adrenaline on the toxicity of local anaesthetics in mice on intravenous infusion at constant rate. Each solution was tested at two rates (black bars = 3.60 ml/hr and open bars = 8.25 ml/hr). $N = 6$.

increased the toxicity more than felypressin. For example the solutions containing 10.0 µg/ml of adrenaline were found to be at least twice as toxic as those containing felypressin the only exception being the procaine combinations which were equally toxic at the highest rate of infusion. The solutions containing 0.5 IU/ml of felypressin were not significantly more toxic than the solutions with 0.05 IU/ml. On the other hand the effect of adrenaline increased with the dose of the vasocon-

strictor. All solutions brought about the common toxic symptoms of local anaesthetics as a result of stimulation and depression of the central nervous system. Solutions containing adrenaline increased salivation and heart rate while the higher felypressin concentration induced diarrhoea and a decrease in the heart rate.

The differences in the effects on the heart rate between felypressin and adrenaline are further illustrated in fig. 5. 0.5 IU/kg of felypressin alone or in combination with a local anaesthetic (prilocaine 200 mg/kg) had no significant effect on the heart rate on subcutaneous injection into mice anaesthetized with pentobarbitone (fig. 5A). A slight bradycardia was observed with a high dose of felypressin (50 IU/kg, fig. 5B). 25 µg/kg of adrenaline had no effect on the heart rate while, as shown in fig. 5C, 100 µg/kg produced tachycardia. Figs. 5D and E show that felypressin did not inhibit the increase in heart rate produced by isoprenaline.

Discussion

Addition of felypressin to some commonly used local anaesthetics brought about a decrease in their toxic effects and a reduction in their plasma levels on subcutaneous injection. Prilocaine and lidocaine were influenced to a greater degree than procaine and tetracaine by the addition of felypressin. This indicates an interaction between the localizing agent and the compound which varies with the type of local anaesthetic used. With the former two compounds the effects of 0.05 IU/ml of felypressin were comparable to those of adrenaline 1:100,000. In accordance with the results of AVANT & WEATHERBY (1960) and ÅSTRÖM & PERSSON (1965) adrenaline was more active in combination with tetracaine and lidocaine than with procaine and prilocaine. The findings suggest that the interactions of felypressin and adrenaline with various local anaesthetics may differ qualitatively.

The findings that the optimum effect of felypressin on retardation of the absorption from subcutaneous depots was obtained with a very low concentration and that the effect decreased when higher concentrations were used, are in agreement with the results of BERLING (1966) in dental plexus anaesthesia. Similar results were also obtained in sciatic nerve block in rats (ÅKERMAN 1966). Apparently this paradoxical effect is due to the synthetic polypeptide *per se* since the preservatives were without any vasoconstrictor action. Felypressin seems to constrict mainly the small vessels and contrary to the catecholamines acts predominantly on the venular side of the microvascular bed (ALTURA, HERSHEY & ZWEIFACH 1965). These authors also observed that felypressin failed to con-

Heart rate beats/min

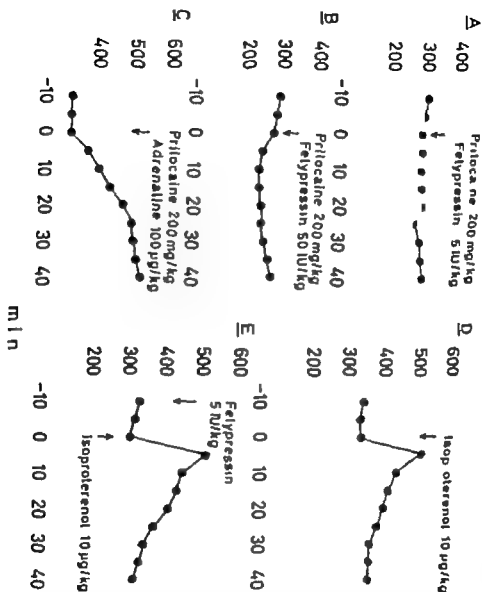


Fig. 5 Effects of prilocaine (0.20 mg/20 g, subcutaneously) with felypressin (A & B) or adrenaline (C) on the heart rate in mice anaesthetized with pentobarbital sodium (80 mg/kg, intraperitoneally). Effect of isoproterenol sulphate (D 10 mg/kg, intravenously) alone and after intravenous pre-treatment with felypressin (E). Each test was carried out on 6 mice.

tract aortic strips and in addition that felypressin inhibited contractions produced by noradrenaline in a dose-dependent manner. The mechanism of action of felypressin is, however, unknown. It is possible that constriction of the microvessels is the cause of the delayed absorption of the local anaesthetics when low concentrations of felypressin are used. Possibly a decrease in the tone of larger vessels by higher amounts of felypressin may be partly responsible for the increased rate of absorption. Further studies on the mechanisms involved in the effects of felypressin on various vascular beds and their interaction with local anaesthetics are needed.

It has been found that contrary to adrenaline, felypressin does not decrease the intravenous LD50 values of lidocaine and prilocaline in mice (ÅKERMAN 1966). In this study felypressin increased the intravenous toxicity of these two agents when large volumes were given by slow intravenous infusion, but the effect was definitely weaker than that of adrenaline. The findings that felypressin increased the toxicity of procaine more than that of lidocaine and prilocaline support the view that the effects of felypressin are dependent on the type of local anaesthetic used. Obviously felypressin *per se* is less toxic than adrenaline since the solutions containing 0.5 IU/ml were not significantly more toxic than those with 0.05 IU/ml while a dose-dependent increase in toxicity was observed following addition of adrenaline. In fact, an appropriate intravenous LD50 value for felypressin has not been obtained despite the administration of several thousand IU/kg (BERDE, WEIDMANN & CERLETTI 1961). 1 IU corresponds to 0.018 mg. This may be compared with the LD50 value for adrenaline, which was found to be about 0.20 mg/kg in mice (ÅSTRÖM, PERSSON & ÖRTENGREN 1965).

The lack of relation between the action of felypressin and the catecholamines on the heart found in this study is borne out by the absence of arrhythmic potential for felypressin (HUGIN 1962 SHANKS 1964 KATZ 1965 KATZ & KATZ 1966). The potential danger of interaction between adrenaline and inhalation anaesthetics such as cyclopropane, trichlorethylene and halothane can therefore be avoided by using felypressin. Conflicting results have been obtained regarding adverse cardiac effects of pure felypressin (MAXWELL 1963 RIBOT *et al* 1963 LONGO *et al* 1964 LIGHT RATTENBORG & HOLADAY 1965). Further analysis of the cause of a possible decrease in cardiac output and the decrease in heart rate after felypressin is desirable.

The results of this study confirm previous observations (BERDE, SCHALCH & DOEPFNER 1964 ÅKERMAN 1966 BERLING 1966) and show that felypressin is capable of reducing the rate of absorption of local anaesthetics. In the present study it has been shown that the optimum

concentration of felypressin varies with the type of local anaesthetic used, as far as both localizing effect and tolerance are concerned. This has been previously found for adrenaline (ÅSTRÖM PERSSON & ÖRTENGREN 1964 ÅSTRÖM & PERSSON 1965). However these effects of felypressin in combination with various local anaesthetics varied independently of those for the catecholamine type of vasoconstrictor. Consequently it is not possible to predict the properties of solutions containing felypressin from the information gathered on the commonly used vasoconstrictor adrenaline. Taking lidocaine and prilocaine as examples, solutions containing felypressin were safer when given intravenously in small animals than those solutions of adrenaline in a concentration which produced the same delay of the subcutaneous absorption. Bearing in mind that great caution should be used in predicting effects in human subjects from experimental results in animals, it would be expected that such combinations will prove to be well tolerated in man. Further studies on potentiation of local anaesthesia by felypressin and the safety particularly the cardiac effects of various combinations seem to be justified.

Summary

The effect of felypressin (octopressin ®) on the acute toxicity of some local anaesthetics was studied in mice.

When injected subcutaneously with sublethal doses of the local anaesthetics, felypressin brought about a decrease in the frequency of the toxic symptoms and a reduction in the duration of the toxic state. Prilocaine and lidocaine were more influenced by the addition of felypressin than procaine and tetracaine. The plasma concentrations of lidocaine and prilocaine were considerably reduced by the addition of felypressin to the subcutaneously introduced solutions. An optimal effect of felypressin was obtained with 0.05 IU/ml in these experiments i.e. about the same as that observed with adrenaline 1:100 000. The localizing effect decreased when higher concentrations of felypressin were used.

On intravenous infusion at different rates, solutions containing felypressin were less toxic than solutions containing adrenaline in a concentration which produced the same delay of the subcutaneous absorption. Contrary to adrenaline, felypressin did not cause a dose-dependent increase of the intravenous toxicity. Felypressin does not seem to have any agonistic or antagonistic effect on the cardiac β -receptors.

The findings suggest that the interactions of felypressin and adrenaline with various local anaesthetics may differ qualitatively. The results indicate that felypressin may be used advantageously as an alternative to vasoconstrictors of the catecholamine type in local anaesthetic solutions.

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Increased Vascular Permeability in Mice Induced by Dextran. A Comparison with the Anaphylactoid Reaction in Rats

By

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VOORHEES, BAKER & PULASKI (1951) first showed that in rats parenteral dextran produces a syndrome with oedema of the paws snout and ears and other signs of cardiovascular disturbances.

This anaphylactoid reaction has since been studied by many workers (review by GIERTZ & HAHN 1966) and there is ample evidence, that dextran elicits the reaction in rats by releasing histamine and serotonin from the tissues, particularly from the mast cells.

There are few publications about the occurrence of dextran reactions in other species. WEST (1957) and ANDERSON & HARDENBERGH (1964) described blueing of the extremities and ears after the intravenous injection of dextran and Evans blue into mice. The latter authors also observed oedema in mice following dextran injection.

It was thought of interest to investigate, whether the NMRI mice used in our laboratory also showed signs of increased vascular permeability (IVP) following dextran injection. Using Evans blue and dextran fractions of varying molecular size, blueing of ears and feet was regularly obtained. These experiments and a study of several factors influencing the IVP are presented here. Finally the dextran reactions in mice and rats have been compared and some aspects of the mode of action in these species of macromolecular histamine liberators of polysaccharide nature are discussed.

Materials and Methods

Animals

Unfasted male albino mice, weight g 20-30 g, from the NMRI strain, and male Sprague-Dawley rats, 200-500 g, were used.

Test compounds

Histamine dihydrochloride and serotonin creatinine sulphate were used and doses refer to the salts. The dextran fractions used were prepared and characterized by Dr. K. Grunsk and her staff of this laboratory either by gel filtration of a solution of hydrolyzed native dextran produced by the *Leuconostoc mesenteroides* strain B 512 (GRUNSK 1964), or by fractional precipitation with ethanol from a solution of hydrolyzed native dextran. M_n is used here for weight average molecular weight. Solutions of polymers or drugs were prepared in 0.9% saline and were given intraperitoneally, subcutaneously, intradermally or intravenously into tail vein, using a thin polyethylene catheter with attached needle. The volume administered was 0.1 or 0.2 ml/10 g mouse.

Procedure

Except for experiments on the duration and onset of the increased vascular permeability and on the relative skin sensitivity to histamine, serotonin and dextran, the following technique was used. Evans blue, 20 mg/kg, was injected intravenously directly following the intravenous injection of solutions of dextran or test colloid. Bleeding of the ears was chosen as criterion of increased vascular permeability and the degree or intensity of bleeding was estimated at 30 and 60 min. following the injection of dextran or test colloid. Two end-points have been used, either singly or in combination: 1) using a quantal response, bleeding - no bleeding, 2) using a graded response, the area of bleeding (degree or intensity of bleeding) being estimated and registered as follows: no bleeding = 0, single patches of bleeding in one or two ears = 1, continuous bleeding along the margin of the ears = 2, bleeding of the whole area of the ears = 3. The mean score of bleeding was calculated for each group of animals.

Onset of the increased vascular permeability

Evans blue, 20 mg/kg was injected intravenously together with 300 or 1,200 mg/kg of dextran M_n 80,000. Estimation of the degree of bleeding was made at 10 minute intervals from 0-80 minutes following the injections. Each point in the graph represents the mean of 10 mice.

Duration of the increased vascular permeability

Evans blue, 20 mg/kg i.v. was injected 0, 60, 120, 240, and 480 minutes after an intravenous dose of 600 mg/kg of dextran. The estimation of the intensity of bleeding was always made 30 and 60 minutes after the injection of Evans blue. Maximal bleeding scores are given in fig. 2, each point in the graph representing the mean of 15 mice.

Relative skin sensitivity: influence of the vascular permeability increasing effect of histamine, serotonin and dextran

0.005 to 50 μ g of histamine and serotonin, in a volume of 0.025 ml, were injected directly under the depilated skin of the back of pentobarbital (30 mg/kg intraperitoneally) anaesthetized mice. In another experiment, histamine 1.0 and 2.5 μ g and dextran M_n 80,000, 3-3,000 μ g, in a volume of 0.05 ml were injected intracutaneously into anaesthetized mice. Immediately thereafter Evans blue, 20 mg/kg was given intravenously and the mice are sacrificed 30 minutes later. After excising the injected skin area, and placing it on a board, the diameter of the blue spots on the inner surface of the skin was measured.

Inhibiting effect of alloxan diabetes

Using a glucose oxidase method (HILLIS & DE VERDER 1963) the blood glucose level in 30 groups of five mice, was found to be 175 ± 20 mg/100 ml blood (mean \pm S. D.). Any

group with values exceeding 215 mg/100 ml is considered as having a significantly ($P = 0.05$ or less) increased blood sugar level. Blood samples were taken by puncturing the retro-orbital vein plexus. For the production of alloxan diabetes, the procedure developed for rats by KLERANOFF & GREENBAUM (1954) was applied to NMRI mice, using a solution of 22.5 mg of alloxan per ml citrate phosphate buffer given subcutaneously in a dose of 225 mg/kg.

Influence of drug pretreatment

A standard dose of 600 mg/kg of dextran M 80,000, followed immediately by Evans blue, was injected 15 min. after the intraperitoneal injection of the test drugs. The average degree of bluing in the drug treated groups was expressed as a percentage of that of the dextran controls tested on the same day.

Oedematogenic properties of dextran fractions of varying molecular size in the albino rat

Rats were used in groups of five. Solutions of dextran fractions were given intravenously into a tail vein in a volume of 0.5–1.0 ml/rat, in doses between 5 and 1000 mg/kg. Observation and registration of oedema and erythema of the paws were made 15–60 minutes after the injections. The degree or severity of the oedema was scored from 0 to ++++. The lowest effective dose was defined as the lowest dose producing oedema of an intensity of +++ to ++++ in at least 4/5 rats.

Vascular permeability increasing effect of dextran after repeated administration

Two intravenous injections of 600 mg/kg of dextran M 80,000 (6% solution) daily were given during three consecutive days. On the fourth day a further injection of 600 mg/kg of dextran was given together with Evans blue and the degree of bluing was estimated.

Effect of pretreatment with polymyxin B or reserpine

Polymyxin B was given twice daily during three consecutive days in single intraperitoneal doses of 15 mg/kg. On the 4th day dextran M 80,000 600 mg/kg intravenously and Evans blue were injected in determine the degree of bluing. Reserpine, in single doses of 2.5 and 12.5 mg/kg subcutaneously were given 16–20 hrs before the dextran. The reserpinized mice were kept in a thermostat box at 27° to prevent hypothermia.

Results

1 Effect of dextran and some other polymers on vascular permeability of the skin of mice

As can be seen from table 1 the two dextran fractions with M_w 40,000 and 80,000 when given in a dose of 1,200 mg/kg, caused marked signs of increased vascular permeability as manifested by intensive bluing of the ears, face, feet, and tails. Six samples of the following three polysaccharides, injected in doses of 600–1,200 mg/kg, proved inactive. The sucrose polymer Ficoll (M_w 28,000–99,000–830,000) hydroxypropylstarch (M_w 100,000–140,000) and hydroxyethylcellulose (approx. M_w 30,000). The following three protein polymers were also tested and found to be inactive: horse serum, bovine serum albumin, and bovine fibrinogen.

Table 1

Vascular permeability in unanaesthetized NMRI mice after intravenous injection of Evans blue and solutions of dextran.

Colloids were given at a standard dose of 1,200 mg/kg intravenously

Colloid	Molecular weight	Bluing of ears, no. reactors/no. injected	Bluing of ears, intensity score (0, 1, 2, 3)
Dextran fraction	40,000	5/5)	III
Dextran fraction	75,000	10/10)	3.0
Controls (saline)	-	0/10	0

) The ears were chosen for estimating the intensity or degree of bluing. Increased bluing was also observed in the feet, face and tails, but quantitation was more difficult at these sites, because of the slight grey-blueish discoloration produced by the Evans blue injection alone. In contrast, the ears remained always uncoloured in controls treated with saline and Evans blue.

2. Onset of the increased vascular permeability following intravenous dextran injection

From the graph in fig. 1 it is evident that a dose of 1,200 mg/kg of dextran M_w 80,000 produced a detectable increase in vascular permeability 10 minutes after injection. The maximal effect was reached 45-50 minutes after injection. With a dose of 300 mg/kg, a vascular response was detected after a latent period of 20 minutes, and a maximal effect was reached 70 minutes after the injection.

3 Influence of molecular weight

From the graph in fig. 2 it is apparent, that the vascular permeability increasing activity of dextran is dependent on molecular weight. Up to a molecular weight of about 10,000 no or very low activity is seen. Maximum activity is shown by fractions with molecular weights between 40,000 and 150,000 and at still higher molecular weights a decrease in activity is observed.

4 Lowest dextran doses producing increased vascular permeability

The results are shown in table 2. No or only very weak vascular permeability increasing activity was found with doses of 1,200 mg/kg in the M_w range up to 11,600. In contrast, the minimal effective dose of dextran fractions within a M_w range of 40,000 and 2,000,000 was found to be 150 mg/kg. For comparison, the minimal doses in rats required to

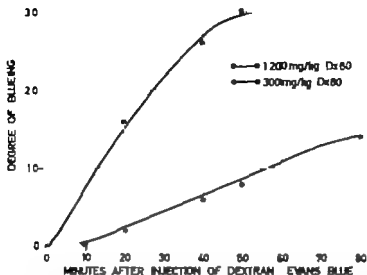


Fig. 1 Onset of the dextran induced increase of vascular permeability of the skin of mice, visualized by blueing of the ears following Evans blue injection. Dextran M 80,000 has been injected intravenously into two groups of 10 mice each, at dose levels of 300 and 1,200 mg/kg.

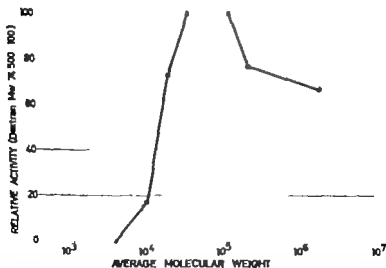


Fig. 2 Relation between molecular weight and vascular permeability increasing activity of various dextran fractions. The fractions were given to groups of 5-10 mice at dose of 1,200 mg/kg intravenously together with Evans blue. The degree of blueing was taken as measure of increased vascular permeability. The activity of the various fractions is given in relation to that of dextran M 74,500.

Table 2

Increased vascular permeability in NMRI mice produced by dextran fractions with varying molecular weight. Approximate minimal effective dose.)

Average molecular weight (M _w) of dextran fraction	660	4 400	11 600	42,000	74,500	250,000	2,000,000	Controls (saline)
Minimal effective dose in mg/kg i.v.	>1,200	>1,200	>1,200	150	150	150	150	

*) A dose of dextran producing at least 3/4 reactors and an average (arcsin) score of 1.0 was defined as minimal effective dose.

produce oedema are given in table 3. It is seen that the lowest oedema producing dose for dextran fractions with M_w between 40 000 and 175,000 is 15 mg/kg. At higher and lower M_w the oedema producing effect decreases, and is practically absent at very high and very low molecular weights.

5 Duration of the increased vascular permeability

From fig. 3 it can be seen that the vascular permeability increasing activity of two dextran fractions with M_w of 40 000 and 141,000 given in

Table 3

Oedematogenic properties of dextran fractions of varying molecular size in the albino rat.

Molecular weight range	3,800	10,500	41,000	60,200	82,600	175,000	2,200,000	30,000,800
Lowest effective dose mg/kg	>1000	60	15	15	15	15	60	>120

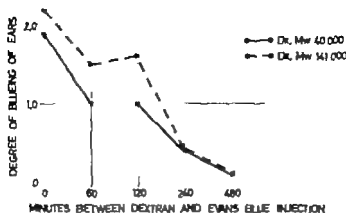


Fig. 3. Duration of the increased vascular permeability in NMRI mice after single intravenous doses of 600 mg/kg of dextran fractions with molecular weights of 40,000 and 141,000.

the same dose, was found to be of comparable duration, the effect subsiding 5-6 hours after the injection. The intensity of the response was somewhat stronger for the higher molecular dextran fraction.

6. Influence of the dextran induced increase of vascular permeability on pentobarbital mortality

When the mortality rate in a group of 41 mice receiving 90 mg/kg of pentobarbital intraperitoneally was compared with that of a corresponding group of mice, receiving an intravenous dose of 2 g/kg of dextran Mw 40,000 fifteen minutes before pentobarbital, no statistically significant difference between the mortality rates of these two groups was obtained.

7. Influence of sensitization with pertussis vaccine on observable signs following dextran injection in mice

In five groups of 6 mice each, injected with 0.25 or 1.0 ml of pertussis vaccine per animal six days before intravenous dextran, no signs of oedema of the ears, snout, paws, or tails were observed following the injection of dextran fractions with Mw 40,000 and 80,000 given in doses of 600 and 1,200 mg/kg.

8. Relative skin sensitivity of mice to the vascular permeability increasing activity of histamine, serotonin and dextran.

The data in table 4 indicate that mice are very sensitive to serotonin, a dose of 0.05 μ g regularly eliciting a blue spot, whereas 0.5 μ g of histamine is required to produce a comparable response on close subcutaneous

Table 4

Relative sensitivity of mice to the vascular permeability increasing effect of histamine and serotonin following close subcutaneous injection.

Measurements of the diameters of the spots were made after injection of Evans blue and excision of injected skin areas.

Histamine, μg injected per spot	50	5	0.5	0.05	0.005
Frequency of blue spots	4/4	8/8	8/8	2/9	0/8
Average diameter per spot in mm	15	10	7	4	0
Serotonin, μg injected per spot	50	5	0.5	0.05	0.005
Frequency of blue spots	5/5	10/10	10/10	5/6	2/6
Average diameter per spot in mm	16	13	10	8	5

Note: 0.025 ml solution per spot was injected.

injection. From table 5 it is evident that dextran is about 3 000 times less active than histamine in producing a blue spot of comparable size, indicating a locally increased vascular permeability.

9 Antagonism of the vascular permeability increasing effect of dextran by pretreatment with drugs

In 16 dextran control groups of five mice each, the average degree of blueing was found to be 1.9 ± 0.36 (mean \pm S.D.). Consequently each drug treated group showing a degree of blueing of 62% or less of that

Table 5

Relative sensitivity of mice to the vascular permeability increasing effect of histamine and dextran, following intracutaneous injection.
0.05 ml solution per spot was injected.

Histamine, μg injected per spot	1.0	2.5	0 (NaCl)	0 (NaCl)
Mean colour intensity	1.3	1.5	0.6	0.5
Average diameter per spot in mm	10	23	3	2
Number of spots	13	8	8	8
Dextran M 80,000 μg injected per spot	3,000	300	30	3
Mean colour intensity	1.3	0.4	0.7	0.4
Average diameter per spot in mm	10	1		1
Number of spots	15	16	8	8

Table 6

Influence of drug pretreatment on the increased vascular permeability (bluing of ears) induced in NMRI mice by dextran M 80,000.

Fixed intravenous doses of dextran and Evans blue were given 15 min. after intraperitoneal injection of test drugs to groups of five mice. Bluing of ears was scored from 30-60 minutes after dextran injection.

Test drug	Dose in mg/kg i p	Degree of bluing of ears in per cent of that of dextran controls	Significant inhibition of bluing, $P = 0.05$ or less)
Chlorpheniramine	30	90	0
Triphenylamine	25	60	+
-	12.5	35	+
-	6	43	+
-	3	74	0
-	1.5	117	0
Diphenhydramine	25	10	+
-	12.5	50	+
-	6	118	0
Promethazine	50	11	+
-	25	33	+
-	12.5	25	+
-	6	109	0
Chlorpromazine	10	0	+
-	5	24	+
-	2.5	35	+
-	1.25	122	0
Acpromazine	5.0	11	+
-	2.5	20	+
-	1.25	45	+
-	0.6	30	+
-	0.3	91	0
Papaverine	50	83	0
Arzidopyrine	100	76	0
-	50	80	0
Calcium gluconate	200	64	±
-	100	90	0
Adrenaline	1.0	0	+
-	0.1	32	+
-	0.02	126	0
Picaterizine	50	29	+
-	25	38	+
-	12.5	57	+
-	6	95	0
BOL 148)	4.0	108	0

) Calculated from the difference between groups treated with drugs and dextran controls tested on the same day *) Bromolysergic acid diethylamide bitartrate.

Table 7

Abolition of dextran induced blooming of ears in mice by repeated previous dextran injections.

Blooming of ears following the 7th dextran injection

Pretreatment	No. of reactors/ no. injected			Degree of blooming, mean score (0, 1, 2, 3)		
	30	60	90	30	60	90*)
6 i.v. dextran (M_w 80,000) injections during 3 days	1/10	2/10	3/10	0.1	0.2	0.3
None (controls)	5/5	5/5	5/5	1.8	2.2	2.4

*) Minutes after dextran injection.

of the controls tested on the same day was considered to possess significantly inhibited blooming ($P = 0.05$ or less).

From table 6 it is seen that among the various drugs tested adrenaline had the strongest protective action with a lowest effective dose of 0.1 mg/kg, followed by the phenothiazine derivatives acepromazine and chlorpromazine with the lowest effective doses of 0.6 and 2.5 mg/kg. Among the antihistamines chlorpheniramine was found to be inactive whereas tripropellamine, diphenhydramine and promethazine proved protective at doses of 6-12 mg/kg. Papaverine, amudopyrine and calcium gluconate had no or a doubtful effect.

The adrenergic alpha blocking agent phentolamine produced protection at a lowest dose of 12.5 mg/kg.

Almost all agents exerting protection, appear to potentiate the vascular permeability increasing effect of dextran at sub-protective doses.

10 Vascular permeability increasing effect of dextran after repeated administration

From the data in table 7 it is apparent that pretreatment with 6 single intravenous dextran injections during three consecutive days strongly reduced the vascular permeability increasing effect of a dose of dextran, usually producing a marked increase in vascular permeability

11 Effect of pretreatment with serotonin and histamine depleters (reserpine and polymyxin B)

The results are shown in table 8. A single injection of reserpine practically abolished the vascular permeability increasing effect of dextran.

Table 8

Effect of pretreatment with polymyxin B or reserpine on the increased vascular permeability induced in mice by dextran, M 80,000.

16-20 hrs after the last dose of the drug pretreatment, dextran and Evans blue were injected intravenously and the degree of bluing was determined.

Pretreatment	Bluing of ears, no. of reactors/ no. injected	Degree of bluing of ears, in per cent of that of controls	Remarks
Six i.p. polymyxin B inject. during 3 days	10/10	96	"transparent"
One s.c. reserpine injection - (2.5)	10/10	58	
(12.5)	2/8	12	
None (controls)	5/5	100	opaque

Note: The polymyxin mice showed the same area of bluing as the controls, but the intensity of the colour was much weaker (transparent) and greenish tinge, not seen in controls, was present.

Pretreatment with polymyxin B during three days only modified the response to dextran, the intensity of the response being decreased, though the area of dye extravasation remained unchanged

Table 9

Inhibiting effect of alloxan diabetes on the increased vascular permeability induced by dextran in NMRI mice.

Scheme 1st day alloxan subcutaneous injection, 2nd day blood sugar determination, 3rd day dextran M 80,000 (600 mg/kg intravenously) + Evans blue, 20 mg/kg intravenously 4th day blood sugar determination.

Exp. no.	Treatment	Blood sugar mg/100 ml 24 hrs before dextran	Blood sugar mg/100 ml 4 hrs after dextran	Bluing of ears, no. of reactors/ no. injected	Bluing of ears, intensity mean score (0,1,2,3)
1	Alloxan 225 mg/kg 48 hrs before dextran	513	512	0/4	0
2	-	386	516	1/5	0.2
3	-	220	not determined	4/5	0.6
4	N Cl (controls)	175	not determined	3/5	2.6

Table 10

Influence of general anaesthesia on the increased vascular permeability induced in mice by dextran M 80,000.

Evans blue and dextran (600 mg/kg) were given intravenously to groups of 10 mice. General anaesthesia was induced and the degree of blueing of ears was registered.

Anaesthetic	Dose in mg/kg i.p.	Blueing of ears, no. of reactions/ no. injected	Blueing of ears, intensity mean score (0,1,2,3)
Urethane	1 800	4/10	0.4
Pentobarbital	60	10/10	2.5
None (controls)	—	10/10	2.2

Note During anaesthesia, animals were kept on a thermostatically controlled table at a temperature of 35

12. Inhibiting effect of alloxan diabetes

As can be seen from table 9 the ability of dextran to produce increased vascular permeability was either inhibited or greatly reduced in alloxan diabetic mice. The diabetic mice were allotted to three groups according to their blood sugar levels. The higher the blood sugar level, the more complete was the inhibition of the dextran induced increase of vascular permeability

13 The influence of general anaesthesia on the dextran induced increased vascular permeability

For results see table 10 Whereas anaesthesia with pentobarbital produced a slight but statistically not significant increase in the dextran induced vascular response, urethane anaesthesia significantly inhibited this response.

Discussion

The blueing of ears, face and feet of mice following intravenous injection of dextran and Evans blue reported here and indicating increased vascular permeability (IVP) of the skin agrees with the findings of WEST (1957) and ANDERSON & HARDENBERGH (1964)

Whereas dextran produces marked anaphylactoid symptoms in most strains of rats, only slight symptoms were observed in mice. ANDERSON & HARDENBERGH found slight oedema of the feet and snout and mild pro-

stration after intravenous injection of dextran M_w 40 000 into unanaesthetized white Swiss mice, as well as increased lethality of dextran treated mice in pentobarbital anaesthesia. In our work, no gross oedema in unanaesthetized NMRI mice, and no significant increase in the lethality of dextran treated mice in pentobarbital anaesthesia have been observed. The varying response of mice to dextran in the two studies is probably due to strain differences.

The IVP seen with dextran is not due to plasma volume expansion as other macromolecules of similar molecular size are inactive at comparable doses. Neither is this vascular response produced by polysaccharides in general as samples of polysacrose, hydroxypropylstarch, and hydroxyethylcellulose of corresponding molecular size were found to be inactive. The structural differences between active and inactive polysaccharides will be discussed later.

The results of this study are in agreement with the findings of WEST (1957), who estimated histamine and serotonin in the abdominal skin of 12 animal species and found that the anaphylactoid dextran reaction, occurring only in rats and mice, was directly related to the presence or absence of serotonin.

In our mouse experiments, the IVP following intravenous dextran developed slowly 50% of the maximal vascular response being reached 20–45 minutes after the injection. The latent period was inversely related to the dose and may reflect the weak mediator releasing action of dextran in mice.

In the rat, intravenous dextran has been shown to release histamine and serotonin in relative amounts of 30:1 from the perfused hindquarter (WEST 1957). Intact rats exhibit an "explosive" release of mediators explaining the rapid onset of symptoms (HALPERN 1956).

The predilection sites of the dextran induced IVP in mice are similar to those of the rat—ears, face and feet. These are regions known to possess a high histamine content in the skin. That the pattern of skin bluing induced by other histamine liberators corresponds to a varying histamine content was demonstrated in the guinea pig by FELDBERG & MILES (1953). In the rat, dextran causes degranulation of mast cells in the predilection sites (ROWLEY & BENDITT 1956; JASMIN 1956). The IVP in mice after intravenous injection of dextran probably starts by degranulation of basophilic leucocytes and mast cells within and adjacent to the blood stream, with subsequent release of vasoactive mediators. When the vascular permeability has increased sufficiently dextran may also reach and degranulate mast cells in the perivascular space. The sequence of events may be analogous to that seen in anaphylactic mast cell disruption in passively sensitized animals after intravenous injection of antigen

Table 10

Influence of general anaesthesia on the increased vascular permeability induced in mice by dextran M 80,000.

Evans blue and dextran (600 mg/kg) were given intravenously to groups of 10 mice. General anaesthesia was induced and the degree of bluing of ears was registered.

Anaesthetic	Dose in mg/kg i.p.	Bluing of ears, no. of reactors/no. injected	Bluing of ears, intensity mean score (0,1,2,3)
Urethane	1,800	4/10	0.4
Pentobarbital	60	10/10	2.5
None (controls)	—	10/10	2.2

Note During anaesthesia, animals were kept on a thermostatically controlled table at a temperature of 35

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Whereas dextran produces marked anaphylactoid symptoms in most strains of rats, only slight symptoms were observed in mice. ANDERSON & HARDENBERGH found slight oedema of the feet and snout and mild pro-

IVP was only partially inhibited by depleting the skin of histamine, by means of polymyxin B. These results suggest, that histamine and particularly serotonin are mediators of the IVP in mice. Similarly the intradermal sensitivity of mice to serotonin is about 23 times higher than that to histamine, comparing activities of the bases.

Among the drugs preventing the dextran reaction in rats, 1-adrenaline and drugs with both antihistamine and antiserotonin effects have proved most effective (STUCKI & THOMPSON 1958). It is very interesting that recent experiments have shown that adrenaline inhibits antigenically induced histamine release from human leucocytes *in vitro* (LICHTENSTEIN & MAROOLIS 1968), particularly in view of the similarities between mast cell degranulation by dextran and the effects of antigen-antibody reactions on mast cells (GOTH 1967).

The same categories of drugs which prevent the dextran reaction in rats also prevented the dextran induced IVP in mice in this study but at 5-20 times lower doses. One explanation for the stronger inhibition exerted by these drugs in mice may be a lower degree of IVP induced by dextran in this species, as indicated by the absence of gross oedema. From the low doses of diphenhydramine and promethazine required to inhibit the IVP and from the inability of these drugs to protect mice from lethal doses of histamine (AMBRUS, PACKMAN ROSSI *et al* 1952), it may be concluded that these drugs exert their inhibitory action by an independent effect on the vasculature, e.g. by preventing contraction of endothelial cells of venules, which MAJNO & LEVENTHAL (1967) believe to be responsible for the vascular leakage following administration of histamine, serotonin and bradykinin. The assumption that an independent effect on the vasculature may be involved is corroborated by the strong positive correlation between drugs inhibiting dextran induced IVP and vasodilator action of the same drugs in mice (RICHTER 1964 CAMPBELL & RICHTER 1967) tripeleminamine being an exception in that it does not have a vasodilator action.

Our failure to inhibit the dextran reaction in mice by the specific serotonin antagonist BOL 148 may depend on the inability of specific serotonin antagonists to abolish actions on the endothelium, or by interference of other mediators.

Pertussis vaccine pretreatment, known to increase the sensitivity of mice to the lethal actions of histamine (PARFENTJEV & GOODLINE 1948) and serotonin (KIND 1957) and to those of a combination of the two amines (BERGMAN & MUNOZ 1968) did not potentiate the dextran reaction in mice. This may imply that the lethal actions of the amines are independent of their vascular effects in mice.

The effect of general anaesthesia on the dextran induced IVP was

negligible, when pentobarbital was used, whereas urethane produced nearly complete inhibition. As the depth of anaesthesia was comparable, the inhibitory action of urethane may depend on its inhibitory effects on enzymes (SEXTON 1953) probably involved in the histamine release by dextran (GOTH 1967).

As to the mode of action of dextran in rats and mice, several findings indicate that dextran may elicit histamine release by interfering with glucose dependent processes in cell membranes. Alloxan diabetes inhibited dextran oedema in the rat (GOTH, NASH, NAGLER *et al.* 1957), and dextran induced IVP in mice in our study. Systemic injections of large doses of glucose and deoxyglucose (GOTH 1959) in rats also diminished dextran oedema.

Admixture of various sugars to dextran abolished its vascular response in rat skin (BERALDO, DIAS DA SILVA & LEMOS FERNANDES 1962; POYSER & WEST 1965) suggesting that residues of simple sugars may compete with those of dextran for the same receptor e.g. on mast cells, thereby inhibiting the release of mediators.

It is interesting, that in dextran and other polyglucoses, known to produce oedema in the rat (yeast mannan, zymosan) the units of the main chain are connected by 1-6 linkages and that highly branched structures of this type have been found more effective than those with a low degree of branching (POYSER & WEST 1965). EDLUND, LÖFQVIST & VÄLI (1952) also stated that dextrans with highly branched molecular chains were more toxic in rats than those with less branched chains. In our mouse experiments, the three polysaccharides which proved inactive did not possess 1-6 linkages in the main chain. In hydroxyethylcellulose and hydroxypropylstarch, the units of the main chain are connected by 1-4 linkages, and in Ficoll, by a random sequence of 1-2, 1-3, 1-4 and 1-6 linkages.

It may therefore be assumed that residues from polyhexoses with 1-6 linkages in the main chain would fit the receptor on the mast cell membrane causing membrane derangements and histamine release.

Recently I found that SVANES (1966) had compared intravenous dextran M_w 40 000 in conscious, anaesthetized normothermic, and anaesthetized hypothermic mice. Only the latter showed an anaphylactoid reaction with oedema and hemoconcentration, possibly due to a prolonged vascular action of serotonin in hypothermia.

Summary

1. Dextran fractions with M_w of 11 600–2,000,000 in minimal intravenous doses of 75–1,200 mg/kg produced increased vascular permeability in the skin of NMRI mice.

2. Compared to rats, dextran exerted a much weaker vascular response in the skin of mice, which may explain the absence of oedema or other pathological symptoms.

3 In the mouse, serotonin depletion, but not histamine depletion inhibited the increased vascular permeability suggesting that serotonin was the more potent mediator of the vascular response.

4 l-adrenaline, phentolamine, and various drugs with both antihistamine and antiserotonin action inhibited the dextran induced vascular response in doses from 0.1–12.5 mg/kg.

5 Experimental evidence suggests that interference with membrane processes involving glucose may explain the amine releasing action of polysaccharides with 1,6 linkages in the main chain.

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Material and Methods

Metabolic studies

Normal white male rats of the State Serum Institute stock weighing 200–250 g were used. The rats were kept in individual metabolic cages. The technique of feeding and quantitative urine collection has been described in previous studies (SKADHAUGE 1966 STEVEN & SKADHAUGE 1969). Briefly the food intake was measured daily and daily rinsing of the cages and the collection of rinsing fluid allowed an almost complete recovery of the urine electrolytes left in the cages. The urine and the rinsing fluid were analysed for sodium, potassium and chloride, and the urine osmolality was measured.

Blood sampling

Blood sampling was performed as in the previous study (STEVEN & SKADHAUGE 1969). In rats, not used for metabolic experiments, but receiving the same food, blood was drawn from the exposed jugular vein while under light ether anaesthesia.

Food, drugs

In all experiments except in the low salt experiment the same batch of Altromin rat powder was used. The salt content of the food changed by varying the electrolyte concentration of an electrolyte solution which was added to the food powder making a paste-like mixture. The resulting salt contents of the diets were as follows: standard salt intake 100, medium salt load: 500, high salt load 1000 $\mu\text{Eq Na/g diet}$. In the low salt intake experiment Altromin "low salt" rat food powder was used. According to the manufacturer the composition of the "low salt" food was: hydrated casein 22%, rice starch 58%, salt free mineral mixture 6%, soya oil 2%, cellulose 4%, vitamins 2%. In this type of experiment the food powder was mixed with demineralised water. The electrolyte contents determined in the low salt intake experiments were: Na 19, Cl 34, potassium 139 $\mu\text{Eq/g food}$. The water content of the food powder was 11%.

Bendroflumethazide (cendyl®; Leo Pharmaceutical Co.) was incorporated in the food in all experiments. A dose of 0.1 mg/g food was given. This is larger than that causing a maximal natriuretic effect (KOSMOSK & KATTE 1960).

Exchangeable body sodium

A separate group of rats was used for these experiments. The rats were kept in individual metabolic cages throughout the experiment. For three days before the experiment the rats received the same diet as during the experiment which was performed as follows. After the cages had been thoroughly cleaned approximately 30 $\mu\text{Ci }^{24}\text{Na}$ in 1 ml isotonic saline solution were injected intraperitoneally into each rat by means of a narrow-calibre Krogh syringe. The syringe was found to deliver its volume with a coefficient of variation of 0.24%. Exactly 24 hours after the ^{24}Na administration, the urine bottles were withdrawn and the cages rinsed with 100 ml demineralised water. The rinsing fluid and the 24 hour old urine were weighed and equilibrium urines subsequently collected for two hours. The activity of the ^{24}Na injected, the equilibrium urines samples, and aliquots of mixed 24 hour urines and the rinsing fluid were measured in the Packard Auto Gamma Spectrometer model 5000. Calculation of exchangeable body sodium (Na_{ex}) was made according to the isotope dilution principle with 24 hour equilibration period

$$\text{Na}_{\text{ex}} = \frac{\text{Cpm } ^{24}\text{Na injected} \sim ^{24}\text{Na lost in the equilibration period}}{\text{Urinary specific activity}}$$

$$\text{Urinary specific activity} = \frac{\text{Cpm/ml "equilibrium" urine}}{\text{mEq Na/ml equilibrium urine}}$$

Sodium scale reading at constant sodium concentration (200 $\mu\text{Eq/l}$)

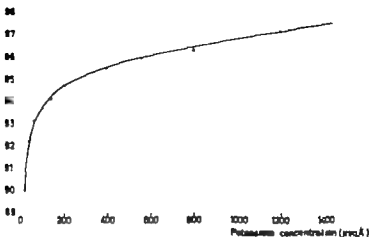


Fig. 1 Sodium scale readings on the Eppendorf flame photometer as function of potassium concentration.

Analyses

The analyses were carried out as in our previous study (STEVEN & SKADHAWON 1969). Since the sodium/potassium ratio in the urine was not constant, the interference of potassium and sodium had to be given special consideration in the flame photometry analyses. In the metabolic experiments standards with approximately the same sodium/potassium ratio as the urine samples were used. In order to correct the urine sodium readings for potassium interference in the isotope dilution experiments in which the Na/K ratios varied greatly the effect of potassium on the sodium scale readings was examined in the following way: Different amounts of KCl (20 μl of 20–1200 mEq/l solutions) were added to 20 ml of a 200 $\mu\text{Eq/l}$ NaCl solution, since this was the approximate concentration to which the urine samples were diluted. The sodium sensitivity of the flame photometer was adjusted to give near maximum scale reading (90) for the NaCl (+ 20 $\mu\text{Eq/l}$ KCl) solution. A curve was drawn by plotting the sodium scale readings on the flame photometer as function of the potassium concentrations (fig. 1). The sodium concentrations were corrected according to the curve. The potassium concentrations in the samples were used as mentioned above, disregarding the minor sodium interference. Most analyses were performed in duplicate. Samples from the first days of drug administration and the first days after discontinuation, were measured in triplicate.

Results

Plasma values

As shown in table 1 long term thiazide administration to normal rats caused a significant decrease in plasma potassium concentration in all experiments. Significant decreases in plasma chloride concentration were observed in the standard salt intake experiment and in the low salt intake experiment. Apart from the medium salt load experiment no change in

Table 1

Plasma osmolality and electrolyte concentrations
(Units: mOsm, and mEq/l)

Diet	Number of rats in each group		Control values Mean \pm S.D.	Thiazide administration Mean \pm S.D.	Difference	Significance
Low salt intake	6	Na	143 \pm 2.65	141 \pm 2.45	2	N.S.
		K	2.69 \pm 0.19	2.19 \pm 0.16	0.50)
		Cl	105 \pm 1.70	98 \pm 2.83	7)
		Os	287 \pm 8	304 \pm 2.00	-1)
Standard salt intake	12	Na	141 \pm 4.25	139 \pm 5.65	2	N.S.
		K	2.86 \pm 0.45	2.40 \pm 0.36	0.46)
		Cl	101 \pm 4.70	94 \pm 3.21	7)
		Os	284 \pm 5	292 \pm 7	2	N.S.
Medium salt load	6	Na	135 \pm 2.79	134 \pm 1.00	1	N.S.
		K	2.60 \pm 0.37	1.78 \pm 0.05	0.82)
		Cl	100 \pm 1.15	98 \pm 2.00	2	N.S.
		Os	289 \pm 4	296 \pm 4	-7)
High salt load	6	Na	158 \pm 2.09	135 \pm 1.00	3)
		K	2.52 \pm 0.15	2.05 \pm 0.24	0.47)
		Cl	96 \pm 3.61	97 \pm 2.23	-1	N.S.
		Os	297 \pm 10	290 \pm 3	7	N.S.

Statistical significance from control

) 0.05 > P > 0.01) 0.01 > P > 0.001) P < 0.001

N.S. denotes not significant.

plasma osmolality was seen, and apart from the high salt load experiment no change in plasma sodium was observed

The effect of long term thiazide administration on the excretion of sodium, chloride and potassium by normal rats receiving different amounts of sodium chloride

1 Low salt intake (19 μ Eq Na/g diet)

After allowing 4 days in the metabolic cages for adjustment to the change in food composition, 6 normal rats were assessed during a 6 day control period before and after a 6 day thiazide administration period. The average daily body weight and urinary excretion of sodium, chloride and potassium are shown in fig. 2. On the first day of administration an

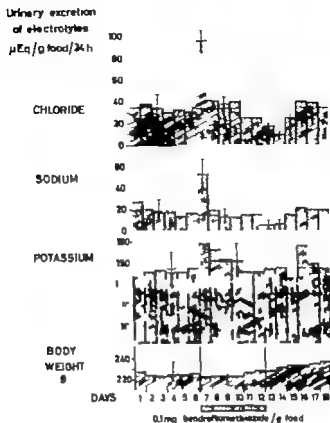


Fig. 2. Natriuretic effect of thiazide administration. Average values and standard deviations for 6 rats receiving the low salt intake.

increase in the excretion of sodium and chloride was seen. A decreased sodium excretion as compared to the control level was seen on the first 2 days, while a decreased chloride excretion was seen during the first 3 days after discontinuation. Apart from these days no change from the average daily control excretion was seen. For each rat the increase in sodium chloride excretion on the first day above the average daily control period excretion value, was calculated and similarly the decrease in the excretion of sodium and chloride on the first 3 days after discontinuation was determined. The average values and the standard deviations of these 4 parameters are shown in table 2. The average value for the increase in sodium excretion on the first day of administration was compared with the average value for the decrease in excretion during the first 3 days after discontinuation by a *t* test. The difference was not significant ($P > 0.1$). Similarly the average value for the chloride loss on the first day of admini-

Table 2

Change in urinary excretion of sodium and chloride(Units $\mu\text{Eq/g food/24 hrs}$)

The increase on the first day of thiazide administration, and the decrease on the first day after discontinuation is compared for the different salt intakes with the standard salt intake values.

Diet		Average value \pm S.D. for the increase in excretion on 1 thiazide administration day	Average value \pm S.D. for the decrease in excretion on 1 day after discontinuation
Standard salt intake	Na	49 ± 34	38 ± 14
	Cl ⁻	78 ± 18	76 ± 18
Low salt intake	Na	35 ± 14 N.S.	26 ± 14 *) 1)
	Cl ⁻	62 ± 18 N.S.	53 ± 18 N.S. 1)
Medium salt load	Na	77 ± 13 N.S.	42 ± 31 N.S.
	Cl ⁻	32 ± 11)	31 ± 68 N.S.
High salt load	Na	35 ± 37 N.S.	63 ± 21 N.S.
	Cl ⁻	-12 ± 31 *)	97 ± 47 N.S.

Statistical significance from control

) $0.05 > P > 0.01$ *) $0.05 > P > 0.001$) $P < 0.001$ N.S. denotes not significant. 1) denotes the first three days after discontinuation.

stration was compared with the average value for the chloride retention during the first 3 days after discontinuation of the thiazide administration. A significant difference was not demonstrated by a t-test ($P > 0.1$). A significant weight loss of 4 g occurred during the thiazide administration ($0.05 > P > 0.02$). There was no change in food intake. Note that after discontinuation 3 days elapsed before complete retention of NaCl occurred. This seems natural as the daily Na intake was smaller than the total amount of sodium retained. Since the aim of the study was to quantitate the total sodium retention, the urinary NaCl excretion after discontinuation in this experiment was calculated for the first 3 days after drug withdrawal.

2. Standard salt intake (100 $\mu\text{Eq Na/g diet}$)

After an initial 3 day control period, 12 rats were given thiazide for 6 days followed by a second control period of 6 days. The results of this experiment have been reported in a previous study (STEVEN & SKADHAUGE 1969). The main data from the experiment have been included in table 2 for comparison. The relevant results are as follows. On the first day of

administration a sodium chloride loss was seen and on the first day after discontinuation a sodium chloride retention was observed. No change from the average daily control excretion was seen apart from these days. The average value for the salt loss found on the first day of administration was compared with the average value for the salt retention on the first day after discontinuation by a t-test. A significant difference was not demonstrated.

3. *Medium salt load (500 μ Eq Na/g diet).*

Six normal rats were studied during two four day control periods i.e. before and after a 5 day thiazide administration period. Control period urine samples were collected during the first 2 days of the initial control period and throughout the second control period. Urine samples from the thiazide period were collected on the first, second and fifth day of administration. For each rat the increases in sodium and chloride excretion on the first day of administration and similarly the decreases in the excretion of these ions on the first day after drug discontinuation were calculated. The average values and standard deviations of these 4 parameters were calculated (table 2). The average values for the increases in sodium and chloride excretion on the first day of administration were compared with the average values for the decreases in excretion on the first day after discontinuation of the drug. No significant differences were found by t-tests (Na $P > 0.1$ Cl $P > 0.1$). There was no change in food intake during the experiment. An average weight loss of 4 g was observed during the thiazide period.

4. *High salt load (1000 μ Eq/g diet).*

After allowing three days in the metabolic cages for adjustment to the change in food composition, 11 normal rats were studied during a 3 day control period before, and during a 6 day control period following a 6 day thiazide administration period. Urine samples were collected each day from 5 rats during the experiment. For the remaining 6 rats urine samples were collected on the last day of the initial control period, on the first and last 2 days of the thiazide administration period and finally during the first three days of the last control period. For each rat the increases in sodium and chloride excretion on the first day of administration and similarly the decreases in sodium and chloride excretion on the first day after discontinuation were calculated. As in the preceding experiment the average values and the standard deviations of these parameters were calculated. No increase in the excretion of chloride was seen. This is

interpreted as a random error due to the limited number of observations. The average value for the increase in sodium excretion on the first day of thiazide administration was compared by a t-test with the average value for the decrease in sodium excretion on the first day after discontinuation. A significant difference was not demonstrated ($0.1 > P > 0.05$), suggesting that the sodium loss on the first day of administration was of approximately the same magnitude as the sodium retention on the first day after thiazide discontinuation. The average values for the decreases in sodium and chloride excretion on the first day after discontinuation of the drug were compared by a t test. No significant difference was demonstrated ($P > 0.1$). Thus a sodium loss was established on the first day of administration, maintained quantitatively throughout the thiazide period and completely restored on the first day after discontinuation of thiazide. The sodium loss on the first day of administration was of the same magnitude as the salt retention on the first day after discontinuation. There was no change in food intake during the experiment.

The potassium excretion in these experiments and in those described in the preceding sections was largely unaffected by the thiazide administration. Any statistically significant change in potassium excretion during or after discontinuation of the thiazide administration did not occur when comparison was made with the excretion during the control periods.

Comparison of the magnitude of the salt depletion caused by long term thiazide administration to normal rats at different levels of salt intake

Up to the present we have found that regardless of salt intake, thiazide administration caused a sodium chloride depletion on the first day. This depletion remained until after discontinuation of the drug, when retention was observed.

In order to assess the importance of changes in daily salt intake on the magnitude of the salt depletion caused by the long term thiazide administration, the average values for the increases in sodium and chloride excretion on the first day of administration in the low, medium and high salt intake experiments were compared by t-tests with those in the standard salt intake experiment. Similar calculations were made for the values from the first day after discontinuation of the drug. As shown in table 2, significant differences from the standard salt intake experiment were not demonstrated for sodium depletion with the exception in the low salt intake experiment, of the decrease in sodium excretion on the first day after discontinuation. The average sodium depletion was therefore calculated for each level of salt intake as $1/2$ (increase in sodium excretion on the first administration day + decrease in sodium excretion on the first

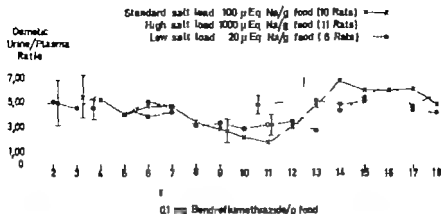


Fig. 3 The effect of thiazide administration on the osmotic urine to plasma ratio. Average values and standard deviations are shown.

day after discontinuation). The following values were found using the values from table 2, (expressed in $\mu\text{Eq/g food}/24 \text{ hrs}$) Low salt intake 31 standard salt intake 54 medium salt load 60 high salt load 50. No significant difference was found for chloride except for the first administration day values in the medium and high salt load experiments.

The effect of long term thiazide administration on the osmotic urine to plasma ratios of normal rats on different levels of salt intake

As shown in fig. 3 long term thiazide administration to normal rats on the low salt intake and the standard salt intake respectively caused changes in the osmotic urine to plasma ratio. The average control period osmotic U/P ratio in the standard salt intake experiment was 4.46 (S.D. ± 1.75) and the average thiazide period value was 2.65 (S.D. ± 0.96). In the low salt intake experiment the average daily control osmotic U/P ratio was 4.98 (S.D. ± 1.81) and the average thiazide period value was 3.19 (S.D. ± 0.75). In each experiment the average control period values were compared with the average thiazide period values by *t* tests. Highly significant differences were demonstrated (low salt intake $P < 0.001$ standard salt intake $P < 0.001$). The average control period osmotic U/P ratio in the high salt load experiment was 4.46 (S.D. ± 0.79) and the average thiazide period value was 4.74 (S.D. ± 0.68). The average thiazide period value was compared with the average control period value. A significant difference was not demonstrated by a *t*-test ($0.1 > P > 0.05$).

Exchangeable body sodium in normal rats at different levels of salt intake

Eleven rats were given the standard salt intake, 7 rats the medium salt

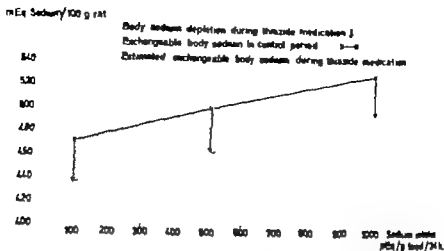


Fig. 4. The effect of different salt intakes on the exchangeable body sodium and on the sodium depletion during long term supra-saltal thiazide administration to the normal rat.

intake and 11 rats the high salt intake. No change in food intake was seen during the experiment. The average values for the exchangeable body sodium were Standard salt intake 4.71 ± 0.71 mEq/100 g rat (Mean \pm S.D.) medium salt load 5.03 ± 1.13 mEq/100 g rat and high salt load 5.29 ± 0.75 mEq/100 g rat. The average increase in exchangeable body sodium from the standard salt intake to the medium salt load amounted to 7% and from the standard salt intake to the high salt load it amounted to 12%. The difference between the average values for the exchangeable body sodium in the standard salt intake and the high salt load experiment as calculated by a *t* test was however not significant ($0.1 > P > 0.05$) due to the large standard deviations. It has been observed that exchangeable body sodium does oscillate (BALDWIN *et al* 1963). For this reason a rather large standard deviation is expected.

In fig. 4 a comparison is given between the observed changes in exchangeable body sodium caused by changes in the daily salt intake and the magnitude of the sodium depletion induced by long term thiazide administration (table 2) when the same salt loads were given to the rats. The sodium depletion (expressed in μ Eq/g food/24 hrs) during thiazide administration was converted to a fall in exchangeable body sodium per 100 g body weight by multiplying by the average food intake and by 100 and dividing by the average body weight. It is seen that an increasing sodium content of the diet seems to increase the exchangeable body sodium, but does not affect the magnitude of the sodium deficit produced and maintained by long term thiazide administration.

Discussion

In this study a 12% increase in exchangeable body sodium seemed to occur when the salt intake was increased by a factor of 10. Below this load, the increase seemed to vary linearly. In rats on a salt intake comparable to our standard salt intake, EDMONDS (1960a & b) found exchangeable body sodium values (4.51 and 4.43 mEq/100 g body weight) very close to our value (4.71 mEq/100 g body weight). After a salt load comparable to our medium salt load, Edmonds found an increase in exchangeable body sodium equal to two thirds of our value. This might be due to the fact that his rats ingested more water. This is well known to increase considerably the glomerular filtration rate thus possibly limiting the increase in exchangeable body sodium. In human subjects JAGGER *et al.* (1963) observed that an increased salt intake augmented the exchangeable body sodium. This shows that body sodium is not an exactly adjusted parameter. In order to function in a satisfactory manner the compensatory mechanisms which lead to the excretion of a large amount of sodium chloride (hormonal and other factors acting on the kidney) apparently need a rather large (and apparently proportional) increase in the regulated parameter.

It might be anticipated that such adjustments could change the renal response to natriuretic agents and the sodium chloride depletion caused by a long term administration. This was not found to be the case in the present study. The sodium chloride depletion was independent of salt intake when this varied by a factor of 50. Thus the two factors influencing exchangeable body sodium—salt intake and natriuretic medication—were independent and additive in their action when applied to the normal rat. These observations are not surprising in the case of the low salt intake. Since the mammalian kidney is an excellent conserver of salt, so that a low salt intake does not deplete the animal of sodium chloride, the fact that a maximal inhibition of the kidney's sodium transport system with a natriuretic agent causes the same depletion of sodium chloride, as when the rat is on a standard salt intake, seems quite natural. When the rat receives high salt loads, however, the natriuretic agents might either be thought not to be natriuretic at all or, on the other hand, to cause a much larger sodium depletion than when the rat receives the standard salt intake. The former possibility is unlikely since even during the highest tolerable salt loads the majority of the filtered sodium is still resorbed in the tubules of the kidney and inhibition is therefore possible. The latter possibility would require that a special standard value of exchangeable body sodium existed corresponding to a zero salt intake to which level the exchangeable body sodium would be brought back,

when the sodium resorption in the kidney was inhibited by a natriuretic drug. This was not found to be the case. The increase in exchangeable body sodium required to bring the salt excretion to a steady state, when the salt intake was increased, was totally uninfluenced by the presence of drug inhibition of renal sodium resorption and the associated compensatory mechanisms. One possible explanation for this finding is that a low sodium diet and thiazide administration, which both reduce exchangeable body sodium do so by independent mechanisms. This speculation is supported by the finding of normal aldosterone levels in the plasma during long term thiazide administration in patients with essential hypertension (BOURGOINTE *et al* 1968). It is possible that there is a special renal compensatory mechanism to drug inhibition which is different from the at least partly aldosterone-regulated adjustment to low salt/high salt intake. Long term sodium chloride loading in the normal rat does not lead to any significant changes in glomerular filtration rate (KLEINMAN *et al* 1965). Thiazide administration also does not change GFR significantly in the normal rat (MORRISON 1962), nor does it, as demonstrated here, change the plasma sodium or chloride concentrations. In the proximal tubule however the local sodium resorption as measured by the split oil droplet method (GERTZ 1963) is decreased during administration of natriuretic agents (ULLRICH *et al* 1966). In spite of this finding, the fractional resorption of sodium might be unchanged or even increased due to an increased tubular diameter and longer passage time (K. H. GERTZ, personal communication 1964; RECTOR *et al* 1966). Thus mechanisms exist which could be mediated by hormonal or other factors different from those inducing changes in sodium excretion, when the salt intake is varied.

If the findings reported here in the normal rat are tentatively extended to the normal human subject on a weight basis, the sodium chloride depletion (50 μ Eq/g food/24 hrs in the rat) induced by a supramaximal thiazide dose would amount to 260 mEq in the normal human subject weighing 70 kg. This NaCl depletion is slightly more than twice the depletion found in the normal human subject (EHRICH 1967) or the human diabetes insipidus patient (SKADHAUGE 1963). Submaximal doses were, however, used in these studies. The increase in exchangeable body sodium when changing from a very low salt diet to a very high salt diet would correspond to approximately 230 mEq in man. This variation is of approximately the same magnitude as the values published by JACOB *et al* (1963). It is interesting to note that a 5-7 times increase in salt load in the presence of thiazide administration is necessary in order to bring the exchangeable body sodium up to control value.

Summary

An increase in salt intake seemed to increase the exchangeable body sodium in the normal rat but the sodium chloride deficit induced by long term maximal thiazide administration was found to be independent of the daily salt intake. Thus it would seem that regulation of exchangeable body sodium during different salt intakes and during long term administration of a natriuretic agent is mediated through independent mechanisms. It was confirmed that the salt deficit induced by the thiazide administration is not restored until after discontinuation of the drug, and that the sodium depletion equals the chloride depletion. In rats receiving low and standard salt intakes a significant decrease in the osmotic urine to plasma ratio was seen during the chronic thiazide administration periods.

Acknowledgement

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Summary

An increase in salt intake seemed to increase the exchangeable body sodium in the normal rat but the sodium chloride deficit induced by long term maximal thiazide administration was found to be independent of the daily salt intake. Thus it would seem that regulation of exchangeable body sodium during different salt intakes and during long term administration of a natriuretic agent is mediated through independent mechanisms. It was confirmed that the salt deficit induced by the thiazide administration is not restored until after discontinuation of the drug, and that the sodium depletion equals the chloride depletion. In rats receiving low and standard salt intakes a significant decrease in the osmotic urine to plasma ratio was seen during the chronic thiazide administration periods.

Acknowledgement

The technical assistance of Miss Aase Frederiksen is greatly appreciated.

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Metabolism in Vitro of ^{14}C Nicotine in Livers of Foetal, Newborn and Young Mice

By

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The passage of nicotine from the mother to the foetus of several species including man has been demonstrated by many investigators (for review see LARSON *et al* 1961) TJÄLVE *et al* (1968), using Ullberg's autoradiographic method have recently studied the passage of ^{14}C nicotine to the foetuses of pregnant mice.

The main route of nicotine detoxication in the body of adult animals is by enzymatic oxidation which occurs in the microsomal fraction of the liver (HUCKER 1960). It was shown that the foetal liver one day before delivery has a much lower ability than the liver of the mother to metabolize nicotine to cotinine (TJÄLVE *et al* 1968). The question of drug detoxication in the livers of foetuses and newborns has recently been the subject of many investigations. No detoxicating activity in the prenatal period and no or very slight detoxication in the neonatal period of life has been shown by most investigators (JONDORF 1958 FOUTS & ADAMSON 1959 HART *et al* 1962 FOUTS & HART 1965). Some activity in the enzymatic system responsible for hydroxylation of zoxazolamine in the rabbit liver before birth was found by DIXON & WILLSON (1968).

The present investigation deals with the alteration in the metabolism of nicotine in livers of mice at different ages before and after birth. The difference in metabolism between males and females and changes in LD50 in relation to age are also studied.

Methods

Experimental animals

White mice of the NMRI-strain were used. The foetuses were delivered by abdominal hysterotomy immediately after sacrifice of the mothers by cervical dislocation, 4, 2 and 1

day before the expected birth. The determination of birth dates for the pregnant mice are calculated on the basis of 19 days as the normal gestation period. Newborn animals were taken just after birth. The ages of the young and adult mice used ranged between 3 and 36 days. The young mice were kept together with their mothers for the first 2 weeks of life when they were used for experiments. The older mice were kept on a standard pelleted diet and received water *ad libitum*.

Compound

Nicotine methyl- ^{14}C was synthesized as previously described (McKENNA *et al.* 1962; HANSEN & SCHWETTLER 1964). The specific activity was 57.33 $\mu\text{Ci}/\text{mg}$. Nicotine bitartrate was used as a source of non-labelled nicotine. 1 g corresponding to 0.329 g of the pure base. The nicotine was dissolved in distilled water (\pm)-cotinine-2- ^{14}C , hydrocotinine and γ -(3-pyridyl)- γ - α -N-methylbutylamide were kindly donated by Dr Herbert McKenna, Jr. Richmond, Va., USA.

Liver preparations

The mice were sacrificed by cervical dislocation and bled by decapitation.

For preparation of the slices the livers were immediately excised and put into bottles containing ice-cold Krebs-Henseleit buffer. Liver slices of approximately 0.3 mm thickness were prepared free-hand with a razor blade.

In order to prepare the supernatant fraction, the livers were removed and rinsed in cold 0.2 M phosphate buffer pH 7.4 and homogenized in two volumes of the buffer mentioned above. The homogenization was carried out with a Waring-blender homogenizer. Livers to an amount of about three grams were pooled and homogenized together for 15 seconds. The homogenates were centrifuged at 10,000 $\times g$ for 20 minutes in a refrigerated centrifuge at $+2^\circ\text{C}$. The supernatant containing the microsomal and soluble fraction of the liver cell was frozen to -20°C and after 20 hours thawed and then used as a source of enzyme. Before use it was diluted with one volume of buffer.

Protein estimations

The protein content of the supernatant was determined according to the Lowry method (SZABOWSKA & KLINCHENKO 1963). Crystalline serum albumin was used as standard.

Incubation

The liver slices prepared were transferred to 25 ml Erlenmeyer flasks containing 2 ml ice-cold Krebs-Henseleit phosphate buffer pH 7.4 with 0.01 M glucose. The amount of slices added ranged between 150 and 160 mg wet weight. For experiments on fetuses and newborn animals, liver slices from 6–10 animals were pooled together to obtain sufficient material. 0.5 μCi of ^{14}C -nicotine was then added to each flask. For each age group at least two incubations were made.

In young and adult mice (36 days old mice) the liver slices from males and females from each age group were incubated separately. Slices from 2–4 animals were used for a single sample. To each bottle 0.22 μCi of ^{14}C -nicotine was added. At least two incubation experiments were performed for each male and female group in all the age groups used. Incubations were continued for 120 minutes in Dubnoff shaker at 37.5°C . During incubation the flasks were covered with a plastic hood. At the beginning and in the middle of the incubation 100% oxygen was flushed through the flasks for 5 minutes. Flasks with no slices were used as controls, throughout the whole procedure.

To the 1.5 ml supernatant fraction kept in a 25 ml Erlenmeyer flask was added 0.5 ml of a

solution containing 25 μ mol nicotine acid anhydride, 37 μ mol $MgCl_2$, 50 μ mol KCl , 0.2 μ mol TPN, 10 μ mol glucose-6-phosphate, in a 0.2 M phosphate buffer pH 7.4. The nicotine in water solution was added in a total volume of 0.5 ml. To reach the necessary substrate concentration non-labelled nicotine bitartrate was added to the radioactive base. The amount of nicotine was calculated as pure base. The flasks were shaken in a Dubouff shaker in an atmosphere of air at 37.5°. The enzymatic processes were stopped by adding 0.7 ml 1 N-HCl. One or two bottles without any co-factors were always run in parallel as blanks. Under the conditions of the assay the enzymatic reaction was linear with time and protein. Linearity was observed for incubations up to at least 25 minutes. The mixtures were incubated for 15 minutes. Substrate curves for a certain age and sex were run with portions of a pooled supernatant mixture representing at least twelve animals. Six different substrate concentrations were made ranging from 0.1–3.2 mM and at least three incubations were made at each substrate concentration. To evaluate the Michaelis constant (K_m) the initial velocity (v) was plotted against $1/[S]$ ($[S]$ = substrate concentration) (HOFMEYER 1957). The function of the best fitting line was determined by the method of least squares and the value of K_m representing the slope of the line was calculated.

Extractions

After incubation the slices were homogenized and the homogenate extracted three times with chloroform-methanol (2:1) (HAMMON *et al.* 1964) or with heptane and chloroform (HUCKER *et al.* 1960), when the radioactive nicotine, cotinine and metabolites present in the water buffer phase are extracted separately. The radioactivity obtained in the different phases by the methods used were determined in a Packard Tri-Carb liquid scintillation counter. Combined mean recoveries of added activity in various fractions ran between 90–95% for the controls and about 80–85% in the case of liver slices, for both methods used.

The contents of the supernatant incubation flask were transferred to a centrifuge flask and cotinine was extracted according to the method described by HUCKER *et al.* (1960). Activity was counted in a Packard Tri-Carb liquid scintillation counter.

Chromatography

The chloroform phase acquired after extraction of homogenized liver slices with chloroform-methanol (2:1) was subjected to chromatography. Thin-layer chromatography (STALE 1962) on silica gel (Kieselgel water w/v 30:60) was used. Plates were activated by heat for two hours. Ammonium acetone benzene ethanol (5:40:50:5) was used as solvent system. The solvent front was allowed to run 1.5 cm (approx. 30 cm.). The plates were exposed to No-screen X-ray film for about 4 weeks. After the development of the film the appropriate spots on the plates were scraped off and activity was determined following addition of scintillation fluid (0.5% PPO + 0.03% POPOP + 4% Aeromil in toluene). After application of the radioactive samples on plates about 90% of the activity could be recovered. Some minute quantities of autooxidative products which appeared on the autoradiograms after incubation with ^{14}C -nicotine under the conditions described were always taken into account. Nonradioactive reference compounds were located by spraying the plates with p-aminobenzoic acid in 95% ethanol (4% w/v) and exposing the plates to cyanogen bromide vapour.

The chromatographic system used separates nicotine and the nicotine metabolites cotinine, γ (3-pyridyl)- γ -oxo-N-methylbutyramide and hydroxycotinine. It has been shown by HAMMON *et al.* (1964) and in the present work that some metabolites formed by mouse liver enzymes co-chromatographed with these references. Two more metabolites not co-

chromatographing with any available reference compound were also observed. In previous studies these still unidentified compounds were called "X" and "Y". The metabolites γ -(3-pyridyl)- γ -oxo-N-methylbutyramide, hydroxycotinine, "X" and "Y" in the following are discussed and presented as "minor metabolites" in all the chromatograms activity was seen at the origin. It is suggested that this activity is also caused by nicotine metabolites (STALHANDSKE, *in press*).

Acute toxicity

The age of the experimental animals purchased on a weight basis was determined according to weight and age curves previously constructed. The experimental mice are divided into 4 groups according to the estimated age 3, 12, 17, 43 and 56 days. Each group consisting of 60-130 animals of both sexes was given 5-7 dose levels of nicotine. From the age of 4 days the sexes were equally represented for each dose. Three days old mice are taken from their mothers just before the experiments. The experiments were performed at room temperature of about 22°C.

Mice were injected intraperitoneally with nicotine bitartrate dissolved in distilled water. Single doses, 1.0, 2.5, 5, 7.5, 10, 12.5, 15 and 20 mg/kg body weight calculated as nicotine base were used. The total volume injected was 0.15 ml for 30 g mice and this was then reduced depending on the weight of the animal. Some groups of mice were given only the corresponding volume of distilled water and served as controls. No deaths were observed in the controls. For the injection of the youngest animals a Hamilton 100 μ l microsyringe was used and the site of injection was pinched to avoid leakage.

The calculations were based on the number of animals dead half an hour after injection. After this time further deaths very occasionally occurred. The kind of convulsions was also observed. The LD₅₀ and its confidence limits for 95% probability were determined by the logarithmic method of LITCHFIELD & WILCOXON (1949).

Results

Metabolism of nicotine in prenatal and early postnatal life

Incubation of ¹⁴C nicotine with liver slices from foetuses and young animals up to the 7th day of life revealed that nicotine is metabolized to a very limited extent in the liver of foetal mice whereas a higher metabolism can be seen in the liver of newborns and during the first week of life metabolic activity increases rapidly (table 1). Only minute quantities of cotinine and other metabolites formed by foetal livers could be detected. The ability of the livers of one week old animals to form cotinine and other metabolites was 25% and 40% respectively of that seen in adult livers.

In order to get more detailed information about the various metabolites, the samples prepared from the same animals were extracted with chloroform-methanol and the chloroform phase which contained most of the radioactivity (table 2) was subjected to chromatographic analysis.

The autoradiogram of a thin-layer chromatogram showing the separated compounds extracted in chloroform phase is presented in fig. 1.

Table 1

Distribution of radioactivity between nicotine and its metabolites after the incubation of ^{14}C -nicotine with liver slices. (Incubation time = 2 hours.) ($8.7 \mu\text{g}$ ^{14}C -nicotine equivalent to 590,000 cpm in each flask.)

Age		Nicotine %	Cotinine %	Water-buffer phase metabolites %
Controls (no tissue)		97.8	1.2	1.0
Days before birth	4	96.6	0.7	2.7
	2	94.6	1.0	4.4
	1	94.2	1.4	4.4
	Birth	92.2	3.9	3.9
Days after birth	3	74.7	10.8	14.5
	7	71.5	12.1	16.4
	Adult (mother)	12.2	49.3	38.5

Only one metabolite was present on radiochromatograms obtained from experiments on animals from two days before birth and up to the third day of postnatal life. The R_f value corresponded to that of cotinine.

The amount of cotinine formed one day before birth was only about 1/ of the radioactivity recovered in the chloroform phase (fig. 2). This

Table 2

Distribution of radioactivity into various chemical fractions after the incubation of ^{14}C -nicotine with liver slices from foetal and newborn mice (incubation time = 2 hours) and extraction with chloroform-methanol (2:1). ($8.7 \mu\text{g}$ ^{14}C -nicotine equivalent to 590,000 cpm in each flask.)

Age		Chloroform phase	Water-methanol phase %
Control (no tissue)		97.8	2.2
Days before birth	4	97.4	2.6
	2	97.3	2.7
	1	95.5	4.5
	Birth	94.7	5.3
Days after birth	3	94.8	5.2
	7	94.6	5.4
	Adult (mother)	81.9	18.1

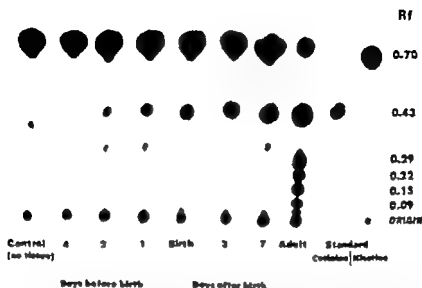


Fig. 1. Autoradiogram of a thin-layer chromatogram of the chloroform phase of extract of livers from foetal and newborn mice. Solvent system: ethanol-acetone-benzene- NH_4OH (5:40:50:5) (v/v). Solvent front: 12.5 cm. Exposure time: 4 weeks. Components: cotinine (R_f 0.70), cotinine (R_f 0.43), γ -(3-pyridyl)- γ -oxo-N-methylbutyramide (0.29), "X" (0.32), hydrocotinine (0.15) and "Y" (0.09). Spots with R_f values between 0.29-0.43 are assumed to be caused by auto-oxidative products.

amount was trippled in newborns and increased gradually during the first week of life. On the 7th day post partum, cotinine represented 11% of the whole activity in the chloroform phase which is about one fifth of the corresponding activity seen in adult animals (fig. 2).

On radiochromatograms of one week old animals three additional weak spots with R_f values corresponding to the minor metabolites, hydroxycotinine "X" and "Y" had appeared. The amount of minor metabolites found was not determined. Some activity was also frequently seen at the origin on all the chromatograms ranging between 0.2-2.0% in prenatal and between 1.7-3.3% in postnatal mice.

For comparison radiochromatograms of incubations with livers of mothers were always run in parallel. On these chromatograms, spots co-chromatographing with cotinine and all the minor metabolites were identified (fig. 1). About 60% of the radioactivity in the chloroform phase was found in spots corresponding to cotinine. The minor metabolites together were responsible for 25% of the activity on these chromatograms (fig. 1).

Radioactivity in the water-methanol phase (table 2) increased rather

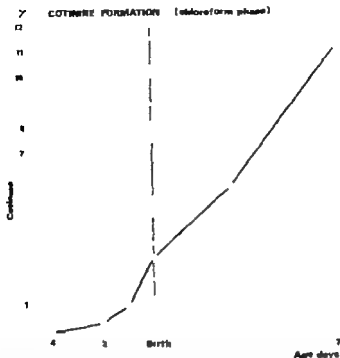


Fig. 2. Formation of ^{14}C -cotinine after incubation of ^{14}C -nicotine with liver slices from foetal and newborn mice of both sexes. (Quantitative data from the autoradiogram in Fig. 1.) Each point on the curve represents the average from at least six livers. The amount of cotinine was calculated as a percentage of the whole radioactivity present in the chloroform phase. The cotinine formed in the livers of adult mice (mothers) amounted to 60% of the radioactivity in the chloroform phase.

slowly during the prenatal and early postnatal period of life. The amount of activity on the 7th day after birth was only slightly higher than that before birth (corresponding to one third of the adult level)

Metabolism of nicotine in young mice in dependence of age

A radiochromatogram of the chloroform phases from experiments, in which liver slices from 14, 21, 28 and 56 days old mice were used, is shown in Fig. 3. As can be seen the liver of the two weeks old mice already possesses the ability though it is very low to form all the metabolites observed in adult animals. Spots with R_f values corresponding to all the minor metabolites were seen on all the chromatograms. The radioactivity of these spots increased gradually with age. Some activity could also be seen at the origin.

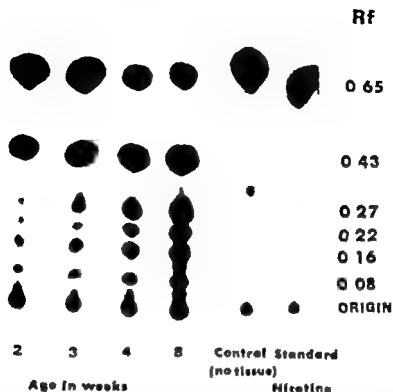


Fig. 3. Autoradiogram of a thin-layer chromatogram of the chloroform phase of extract of livers from mice 2–8 weeks old. Solvent system: ethanol–acetone–benzene–conc. NH_4OH (5:40:50:5) (v/v). Solvent front 1.5 cm. Exposure time 4 weeks. Components: nicotine (R_f 0.65), cotinine (0.43), γ -(3-pyridyl)- γ - α -N-methylbutyramide (0.27), "X" (0.22), hydroxycotinine (0.16) and "Y" (0.08). Spots with R_f values between 0.27–0.43 are assumed to be caused by auto-oxidation products.

Quantitative estimation (fig. 4) revealed that cotinine is the major metabolite observed at all the ages. The formation of cotinine increased with age up to the fourth week of life. The steepest increase in the formation of cotinine was observed between the third and the fourth week. The amount of cotinine found at eight weeks was somewhat lower than at four weeks.

The minor metabolites observed at two weeks represented less than 0.7% of the activity present in the chloroform phase. This activity increased to 1.5% at three weeks and to 8% and 23% at four and eight weeks respectively (fig. 4). The amount of activity at the origin ranged between 2.1–3.2%. The disappearance of nicotine gradually increased with age up to eight weeks even in the absence of any increase in cotinine.

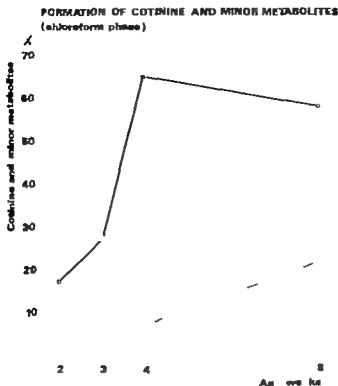


Fig. 4. Formation of cotinine (O—O), γ -[3-pyridyl]- γ -oxo-N-methylbutanamide, hydroxycotinine, X" and "Y" metabolites (● ●) after incubation of ^{14}C -nicotine with liver slices from young mice of various ages of both sexes. The figure shows quantitative data in the autoradiogram in fig. 3 expressed as percentage of the whole radioactivity present in the chloroform phase. Each point on the curves represents the average from at least four livers. The percentage of minor metabolites was counted as total of all the minor metabolites formed.

formation between four and eight weeks (fig. 5). This was due to the great increase of other metabolites between four and eight weeks (fig. 3 and fig. 4).

The activity recovered in the water-methanol phase was more than trippled between 2 and 8 weeks (from 4.8% to 17.4% of the whole activity recovered in both phases) (table 3).

The results obtained with the use of the extraction method (HUCKER *et al.* 1960) when the whole amounts of nicotine, cotinine and water phase metabolites were extracted separately confirmed that there was a rapid increase in formation of cotinine with increasing age and a concurrent decrease in the amount of non-metabolized nicotine.

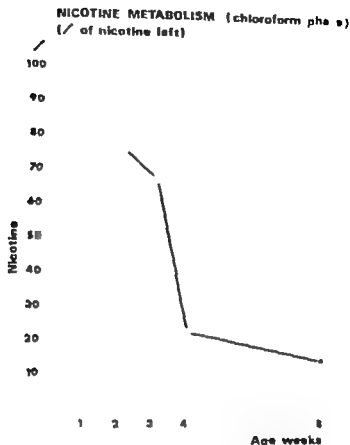


Fig. 5 Metabolism of ^{14}C -nicotine after incubation with liver slices from mice of various ages and of both sexes. (Quantitative data in the autoradiogram in fig. 3.) Each point represents the average from at least four livers.

Cotinine formation in supernatant fractions of mice liver in dependence of age and sex

Formation of the major metabolite cotinine was measured quantitatively in the 10 000 \times g supernatant fraction of livers of mice at various ages and of both sexes.

Substrate curves made with the supernatant from two to eight weeks old mice revealed that saturation of cotinine forming enzymes is approached at a substrate level of 1.6 mM nicotine.

No clear differences between the amounts of cotinine formed by males and females were observed except at four weeks of age (fig. 6). At this age females formed somewhat higher amounts of cotinine at each substrate level (fig. 7) than males.

To test the significance of this difference, another series of incubations

Table 3

Distribution of radioactivity into various chemical fractions after the incubation of ^{14}C -nicotine with liver slices from young mice (incubation time — hours) and extraction with chloroform-methanol (2:1). ($3.8\text{ }\mu\text{g}$ ^{14}C -nicotine equivalent to 260,000 cpm in each flask).

Age weeks	Chloroform phase %	W ater-methanol phase %
Control (no tissue)	97.8	2.2
2	95.2	4.8
3	92.8	7.2
4	88.7	11.3
8	82.6	17.4

with different preparations of supernatants was made at a substrate concentration of 1.6 mM of nicotine. This study also showed a higher amount of cotinine formed by females, but the difference observed was not significant ($P > 0.4$).

The supernatant fraction of livers of one week old mice has a very low ability to form cotinine (fig. 6). The amount of cotinine formed at the substrate concentration of 1.6 mM is only about 12% of the amount seen in the adult. No increase of cotinine forming ability was seen between the ages of one and two weeks. After two weeks of age the ability to form



Fig. 6. Cotinine formation at different ages and in both sexes. \bigcirc — \bigcirc females, \bullet — \bullet males. Nicotine in concentration of 1.6 mM was incubated for 15 minutes with 10,000 μ supernatant fraction of mice livers. Co-factors were added as stated in methods. Each point represents mean of at least 8 incubations with pooled supernatant fractions representing 1 to 35 livers.

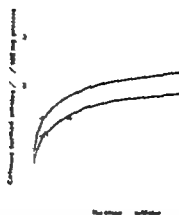


Fig. 7 Relation between substrate concentration and rate of cotinine formation from nicotine in 4 weeks old male (Δ — Δ) and female (Δ — Δ) mice. Nicotine was incubated for 15 minutes with a 10,000 \times g supernatant liver fraction. Portions of the same male and female fraction each prepared from 16 litters were used in all the incubations. Co-factors were added as stated in methods. Each point represents mean of at least three incubations.

cotinine starts to increase rapidly. The increase is most pronounced between 3 and 4 weeks of age. At four weeks of age the amount of cotinine formed approaches the adult level.

Calculations obtained from substrate curves reveal that the K_m value decreases from 2 to 4 weeks and that the adult K_m value is higher than that seen at four weeks (table 4).

Acute toxicity of nicotine in dependence of age

The LD₅₀ of nicotine after intraperitoneal injection of mice at various ages is shown in table 5.

Table 4

Metabolism of nicotine to cotinine in the 10,000 \times g supernatant fraction of livers from 2 to 8 weeks old mice. K_m values are calculated from the combined results of separate experiments with males and females. For conditions and number of experiments, see methods.

Age	K_m (mM)	r
2 weeks	0.66	< 0.01
3 weeks	0.37	< 0.01
4 weeks	0.10	< 0.01
8 weeks	0.21	

Table 5

Acute toxicity of nicotine in young and adult mice.

Mice (days)	No. of mice	Mean body wt. (g)	Acute toxicity I.p. LD50 (mg/kg body wt.)
3	80	2.7	11.2 (9.7-12.8)
12	60	5.8	2.7 (1.4- 5.2)
17	60	8.2	4.4 (2.6- 7.2)
24	170	13.4	9.3 (8.5-10.2)
35	130	19.4	11.5 (10.0-12.9)
56	120	29.6	12.5 (10.8-14.5)

Figures in brackets are 95% confidence limits.

The LD50 of nicotine at 3 days (11.2 mg/kg) was of about the same order as at 56 days (12.5 mg/kg). At 12 days of age when the mice were most sensitive to nicotine, the LD50 was 2.7 mg/kg. From 12 days to 56 days of age the LD50 increased from 2.7 to 12.5 mg/kg. No significant difference in LD50 between 35 and 56 days was observed.

The lethal doses in mice of 12 to 56 days of age always produced convulsions followed by death 2-5 min. after the injection. The pattern of convulsion corresponded to that described in previous works (excitation, tremor jerking movements, clonic and tonic convulsions and death) (YAMAMOTO *et al.* 1966). The character of the seizures differed in 3 days old mice. With lethal doses trembling and hyperkinesia occurred which was followed by loss of body posture and finally by slow but intensive paddling movements of the legs. Respiratory rate was lowered with periods of apnoea and the animals successively became more cyanotic and in the preagonal stage they were also very pale. Death in most cases did not occur until 10 minutes after the injection. Contrary to adult animals no distinct clonic or tonic convulsions were seen.

Discussion

The foetal mouse liver has the ability to metabolize nicotine to cotinine on the last two days of foetal life. The cotinine formation starts to increase after birth and the metabolism of nicotine rapidly increases with age.

The metabolism of drugs in the microsomal fraction of animal liver where the nicotine is also metabolized (HUCKER *et al.* 1960) is very limited in new born and foetal animals as compared with adults (FOOTS & HART

1965 CONNRY & BURNS 1962) Only a few drug metabolic pathways have been detected in the last stage of foetal life — i.e. conjugation in mice and rats (DUTTON 1966) and reduction in rabbits (HART *et al.* 1962). With regard to the oxidative pathways, hydroxylation of zoxazolamine was found in the last two weeks of foetal life in rabbits (DIXON & WILLSON 1968). All the enzymatic activities seen were, however, very limited as compared with adults. On the other hand side chain oxidation of hexobarbital and N-dealkylation of aminopyrine could not be detected by HART *et al.* (1962) or DIXON & WILLSON (1968).

The apparent changes in differentiation of the endoplasmic reticulum in liver cells of foetal mice with the appearance of an endoplasmic reticulum devoid of ribosomes on the 16th day of foetal life (PETERS *et al.* 1963) can be related to the beginning of a drug metabolizing activity which is mainly localized in the smooth-surfaced part of endoplasmic reticulum (FOOTS 1961).

The increase in nicotine metabolism observed after birth and the gradual metabolic increase with age is in agreement with the observations made for some drugs by various investigators (FOOTS & ADAMSON 1959 JORDNORF *et al.* 1958 HART *et al.* 1962). Of particular interest is the steep increase in nicotine metabolism between the 3rd and the 4th week of age. This period of life coincides with the cessation of weaning and the general expansion of physical activity in the mouse. A similar observation was made by CATZ & YATTE (1967) who showed a significant increase in hexobarbital degradation at this period of life in mice.

After the 3rd week a higher amount of other metabolites in addition to cotinine appears, suggesting the appearance of further pathways in nicotine metabolism (MCKENNIS *et al.* 1962 BOWMAN *et al.* 1963). It is possible that the metabolism of cotinine after the 4th week also increases as the *in vitro* system used allows the metabolism of cotinine during incubation (MORSELLI *et al.* 1968).

In the present work a somewhat higher formation of cotinine by females as compared with males could be seen at 4 weeks of age. However this difference was not significant. Our results are in accordance with the findings of QUINN (1958) KATO (1962) and NOVICK (1966), who failed to observe any sex differences in the metabolism of drugs in mice. In contrast sex differences in drug metabolism in mice were found by CASTRO & GILLETTE (1967). The different strains of mice used in various studies could partially explain these discrepancies (CASTRO & GILLETTE 1967). In rats the sex difference in the metabolism of drugs also varied according to the substrate used (KATO & GILLETTE 1965).

The development of nicotine metabolism is very likely not only a matter of an increasing net content of nicotine metabolizing enzymes.

The observed change of K_m values with age indicates that the development also involves a change in substrate affinity

The LD50 values obtained in our study with adult animals agree with those reported by previous authors (for review see LARSON *et al* 1961). The acute toxicity of newborn mice does not seem to have been studied previously and it was surprising to find that the LD50 value of 3 days old mice was approximately the same as for adults.

With the exception of the neonates the mice show a decreasing sensitivity to the acute toxic effect of nicotine with increasing age. The difference between various ages might depend on differences in absorption, distribution, excretion and metabolism of nicotine or differences in the sensitivity of physiological receptors. From the present results the toxicity seems to vary directly with the development of enzymatic metabolic activity from 2 to 8 weeks

The cause of death in acute nicotine poisoning is not exactly known. Most investigators agree that death is the result of respiratory arrest (for review see LARSON *et al* 1961). Opinions are divided as to whether this arrest is primarily provoked by central depression or by peripheral paralysis. Regardless of whether the central or peripheral effect is of primary importance, the seizures evoked centrally (for review see LARSON & SILVETTE 1968) undoubtedly contribute to the fixation of the respiratory muscles and to the increased oxygen consumption (KING 1966)

In neonates some other factors must be involved as they tolerate doses as high as adults despite the much lower capacity to metabolize nicotine.

It has been shown that several convulsant drugs have a weaker activity in newborn and very young animals (PYLKKE & WOODBURY 1961; KOBAYASHI 1963; FERNGREN 1965) as compared with the adults. The higher resistance of the newborn to the hypoxic states is also known (for review see DONE 1964). KOBAYASHI (1963) showed that the histological maturation of the brain cortex is low and that spontaneous EEG activity is absent in mice during the first week of life. He also failed to induce EEG activation and convulsions during this period with a drug active in this respect in older mice. The failure to induce distinct clonic or tonic seizures by nicotine in neonates in the present investigation suggests that the immaturity of the CNS is one of the reasons for the lowered response of the very young organism to the toxic effect of nicotine.

Our findings could also be interpreted as an indication of a certain lack of nicotine "receptors" at birth. Some time after the 3rd day however such receptors seem to develop but at this time, the enzymatic systems responsible for the detoxication of nicotine, are not sufficiently active. These two facts could explain the marked increase in nicotine toxicity 12 days after birth.

Summary

The metabolism of nicotine in the livers of foetal young and adult mice has been studied *in vitro*. The metabolism of nicotine in the foetal liver with the formation of cotinine was seen at the end of foetal life. Cotinine was found to be the major metabolite at all the ages investigated. After birth the ability of the liver to metabolize nicotine increased and approached the adult level at four weeks of age. The LD₅₀ value for intraperitoneally injected nicotine increased with age in two to eight weeks old mice. A relation between the rate of metabolism *in vitro* and the lethal toxicity of nicotine in young mice was observed. However, despite a very low metabolism of nicotine in the livers of three days old mice the LD₅₀ at this age was almost of the same order as for adult mice. In three days old mice, nicotine did not induce the convulsive pattern seen in adult mice.

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The authors are grateful to the Swedish Tobacco Company and the American Medical Association Education and Research Foundation for supporting this investigation. The authors are also indebted to Karl och Alice Wallenbergs Stiftelse for general equipment for isotope research work. We wish to thank Mrs. A. Österblom, Mrs. G. Mabo and Mrs. B. Hagtorn for their skilled technical assistance.

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The Physiological Mechanism Involved in Hangover

1. The Oxidation of Some Lower Aliphatic Fusel Alcohols and Aldehydes in Rat Liver and their Effect on the Mitochondrial Oxidation of Various Substrates

By

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The cause of the "hangover" that follows the heavy consumption of alcoholic beverages has not been finally elucidated. Based on their observation that the ethanol of different distilled spirits was metabolized at different rates, HAGGARD GREENBERG & COHEN (1943) suggested that congeners present in various amounts in the beverages act as inhibitors of the metabolism of ethanol in the liver. Thus the amounts of various congeners present may explain the differences in the after-effects of different alcoholic beverages. In opposition to this explanation MURPHY, GREENBERG & CARROLL (1967) investigating the acute after effects of vodka, bourbon and an artificial superbourbon (congeners added), observed that the congeners themselves have a direct, long lasting effect on the central nervous system. BRUSCH CERRATO PAPAS & STRACCIA (1955) came to the same conclusion.

Investigations by SNELL (1957) SIHTO, NYKÄNEN & SUOMALAINEN (1962), SUOMALAINEN (1965) MURPHY *et al.* (1967), SUOMALAINEN & NYKÄNEN (1968) and NYKÄNEN PUPUTTI & SUOMALAINEN (1968a & b) have shown that distilled spirits, such as whisky cognac and bourbon, contain large amounts of congeners. The congener present in the largest amount is the fusel oil, which is mainly composed of *n*-propanol, isobutanol and isoamyl alcohol BERGGREN (1938), WALLGREN (1959), and MACGREGOR, SCHÖNBAUM & BIGELOW (1964) have reported that the lower aliphatic alcohols become increasingly toxic with increasing carbon chain length. This is in accordance with the observation by HAGGARD MILLER & GREENBERG (1945) that the amyl alcohols are twelve times

more toxic than ethanol. They also found that the administration of amyl alcohols depressed the animals long after the point at which all the amyl alcohol and its corresponding aldehyde had disappeared from the blood. They suggested that this long sedation was due to the valeric acid, which is known to have this effect.

LEON & MUNOZ (1938) have shown that ethanol inhibits the tricarboxylic acid cycle thereby preventing the acetate produced from being further oxidized. Aliphatic alcohols other than ethanol, have also been shown to inhibit the oxidation of acetate (MAJCHROWICZ & QUASTEL 1961). In addition, FORSANDER (1967) showed that the organic acids formed from other aliphatic alcohols are not oxidized further but are accumulated, as the tricarboxylic acid cycle is inhibited by these alcohols. These observations may explain the severe intoxication on administration of amyl alcohols in terms of the accumulation of valeric acid.

The rates of metabolism of the fusel alcohols as well as the effect of these alcohols and their corresponding aldehydes on the metabolism of ethanol and acetaldehyde were therefore chosen as the main subjects of the present work. Furthermore we have determined the intracellular distribution of the aldehyde dehydrogenase.

In previous papers by REIAK & TRUITT (1958), BEIR & QUASTEL (1958) and KIESSLING (1962a, b c & 1963) acetaldehyde was shown to be a rather potent inhibitor particularly of the mitochondrial oxidation of pyruvate. In the present paper these studies have been extended to include the inhibiting effect of the aldehydes formed from the fusel alcohols present in alcoholic beverages.

Experimental

Tissue preparation

Adult Wistar rats from this laboratory's stock were used in all experiments. Some animals were killed by decapitation and the livers were chilled immediately in ice-cold buffer solution. The homogenate was prepared by homogenizing *liver* for one minute with four volumes of ice-cold 0.1 M potassium phosphate buffer (pH 7.3) in Potter-Elvehjem glass homogenizer. To minimize the nicotinamide-adenine dinucleotide activity nicotinamide was added to the buffer immediately before homogenization to give a final concentration in the suspension of 0.03 mM (LINQVIST, SWENSSON & PETERSEN, 1967). The cell debris was removed by centrifugation at $800 \times g$ for 5 minutes.

The rats of which livers were to be perfused were anaesthetized by the intraperitoneal injection of 5 mg of nembutal (*mebumalium* NFN, Abbot, J. A., Belgium) per 100 g of body weight. Nembutal is an anaesthetic that has been shown not to interfere with the metabolism of ethanol (EDWARDS 1940). A thin plastic tube, coming from an artificial lung, was joined to the hepatic portal vein, and another thin plastic cannula, ending in blood pool, was joined to the hepatic vein.

The mitochondria were isolated by the method of HANSTEN & LÖW (1955). The medium used was 0.25 M sucrose-EDTA (0.11 mM with regard to EDTA). In the experiments

designed for studying the intracellular distribution of the aldehyde dehydrogenase, the method of preparing mitochondria was slightly different. The homogenate obtained after centrifugation at 800 x g was divided into two equal samples. One sample was incubated without further centrifugation and the other sample was centrifuged at 9000 x g for 10 minutes; the supernatant was re-centrifuged in the same way and the two mitochondrial pellets were pooled and washed once with ice-cold 0.25 M sucrose-EDTA (see above). Finally the washed pellet was suspended in 0.25 M sucrose to the same volume as that of the supernatant free from mitochondria. Thus three liver samples of the same volume were obtained, first, the supernatant free from mitochondria, second, the mitochondria free from supernatant and, third, the homogenate containing both supernatant and the same amount of mitochondria as in the second sample.

Incubation and analysis

The metabolism of various aliphatic alcohols was studied in liver homogenate and in liver *in situ* by the perfusion method described by FORSANDER, WALLINEN & SUOMA-LAHTI (1960). To the liver homogenate the following additions were made in Erlenmeyer flasks: 5 ml of liver homogenate (0.25 g liver/ml of homogenate), 1 ml of 0.1 M K_2HPO_4 -HCl buffer, alcohol and NAD (Sigma, grade III) to final concentrations of 11 and 2 mM respectively and in some incubations pyrazole (final concentration 2 mM). In some experiments ethanol was incubated, as mentioned above, in the presence of another lower aliphatic alcohol (concentration ratio 100:1). The final volume was 73 ml. The flasks were sealed with paraffin to minimize evaporation. Flasks with the same additions but with 5 ml of the phosphate buffer instead of homogenate were run parallel to the liver incubations, in order to facilitate correction for a possible loss of alcohol by evaporation. The incubations were performed at 30° with mechanical shaking.

Samples (0.5 ml) were taken at zero time and at intervals of 15 minutes, immediately deproteinized by the addition of 0.5 ml of ice-cold 10% perchloric acid and centrifuged. Excess perchloric acid was precipitated by the addition of 2 M KOH to pH 7 and removed by centrifugation. The samples were analyzed by gas chromatography. The gas chromatograph was Varian Aerograph 600 C equipped with flame ionization detector. The carrier gas was nitrogen. The column was packed with 10% carbowax 1000 on Chromosorb W 100-120 mesh.

When the liver-perfusion technique was used, the prepared rat (males weighing 300-400 g) was placed in physiological saline bath thermostatically controlled at 37°. The blood was heparinized *in vivo*; blood drawn from members of the staff of this laboratory. Blood and physiological saline were mixed in equal proportions and used as the perfusion medium (200 ml), to which a known amount (5.3 mmoles) of the alcohols was added. During the experiments the blood flow through the liver was about 2 ml per minute. The liver was perfused for 60 minutes, with sampling every 15 minutes. Then, in order to estimate the evaporation of alcohol from the perfusion system, the liver was unclamped by connecting the two tubes and further samples taken every 15 minutes for 75 minutes. The samples were treated and analyzed as described above.

In the *in vivo* experiments four groups of rats were given intraperitoneal injections as follows: group 1, 3.41 mmoles of ethanol per 100 g; group 2, ethanol, as mentioned above, mixed with 0.35 mmoles of n-propanol per 100 g; group 3, ethanol, as above, mixed with 0.32 mmoles of isobutanol per 100 g; and group 4, ethanol, as above, mixed with 0.32 mmoles of lauryl alcohol per 100 g. Blood samples (0.10 ml) were withdrawn from tail vein after 15, 45, 75 and 105 minutes and then after every 60 minutes. The samples were deproteinized and analyzed by gas chromatography as described above.

The intracellular localization of acetaldehyde dehydrogenase was determined by incubating four vessels with the following: 4 μ moles of ATP, 33 μ moles of $MgCl_2$, 10 μ moles of phosphate, 50 μ moles of glucose, 13.2 μ moles of acetaldehyde, 0.95 μ moles of NAD, 4.4 μ moles of pyrazole (to inhibit ADH activity in the homogenate), 125 μ g of hexokinase (Sigma, grade III), and 0.25 M sucrose to a final volume of 3 ml. The pH was 7.4, which, according to WALKMEYER & WETTERHOUSE (1953), is an optimal pH for the aldehyde dehydrogenase. Either 1 ml of homogenate, 1 ml of supernatant, 1 ml of mitochondrial suspension (mitochondria from 0.3 g of liver per ml) or 1 ml of 0.25 M sucrose was added. The preparation of these subfractions has been described above. The last flask was used to estimate the disappearance of acetaldehyde by evaporation only. Zero samples were taken, the flasks were sealed and incubated and samples were taken every 15 minutes until the 60th minute. The samples (0.3 ml) were deproteinized and analyzed as described above.

Mitochondria prepared in the same way were used when the mitochondrial oxidation rates of various aldehydes were studied. In this case the Warburg method, the gas chromatographic method, and an oxygenographic method (platinum micro-electrode) were used to estimate the oxidation rate. Proteins were determined according to CLELAND & STAIRS (1953) by a colorimetric method depending on the protein bluret reaction. When the oxygen utilization was measured by the platinum micro-electrode (G.M.E., model KM), the additions were as follows: 1.2 ml of 0.25 M sucrose, to which had been added 8 μ moles of Tris and 20 μ moles of potassium phosphate (pH 7.3), 0.1 ml of mitochondrial suspension (mitochondria from 1 g of liver per ml), 6 μ moles of aldehyde and 0.1 μ moles of ADP.

The oxidation rate of acetaldehyde in the presence of other aldehydes was studied in E. Konneker flasks as follows: 13.2 μ moles of acetaldehyde, together with the same amount of one of the other aliphatic aldehydes to be studied, ATP, $MgCl_2$, phosphate, glucose, sucrose, NAD, hexokinase and mitochondria, as mentioned above, were incubated and samples were taken and analyzed by gas chromatography as described earlier.

The aldehydes as inhibitors of the mitochondrial oxidation of substrates were studied by measuring the oxygen consumed in the absence and in the presence of the aldehydes by the Warburg method or the oxygenographic method described above. 30 μ moles of substrate and 3 μ moles of aldehyde (Warburg experiments) or 10 μ moles of substrate and 1.5 μ moles of aldehyde (oxygenographic experiments) were added to the mitochondrial system (see above), shown in table 5.

Results

Oxidation of aliphatic alcohols

Incubating short-chain aliphatic alcohols with a rat liver homogenate and in a rat liver perfusion system revealed varying rates of metabolism of the alcohols (fig. 1). The rate of metabolism increased in the succession isomyl alcohol, ethanol, isobutanol and n-propanol. It was also found that the alcohols at the concentration used were metabolized at constant rates. Liver homogenates from male and female rats oxidized the alcohols at equal rates.

Alcohols with three or more carbon atoms may, as mentioned above, occur in small amounts as by-products in alcoholic beverages. It was therefore of interest to study the effect of these fusel alcohols on the metabolism of ethanol. Thus these alcohols were added in low concentra-

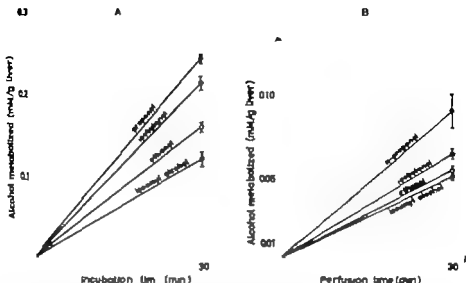


Fig. 1. Rate of metabolism of lower aliphatic alcohols in rat liver homogenate and in perfused rat liver. A. *1 ml* of rat liver homogenate (0.25 g liver per ml) was incubated with 80 μ moles of alcohol. Final incubation volume was 7.3 ml. B. *1 ml* of 5.3 μ moles of alcohol in 200 ml perfusion medium (human blood) was perfused through rat liver. The alcohol present after 30 minutes of incubation was estimated by means of gas chromatography. The curves are mean values of 3-10 experiments. The bars show the standard errors.

tions to liver homogenates, together with ethanol. The results, given in table 1 show that the presence of the aliphatic fusel alcohols diminished the rate at which ethanol was metabolized. Furthermore the fusel alcohols were metabolized simultaneously.

Addition of the ADH inhibitor pyrazole inhibited the metabolism of all aliphatic alcohols to the same degree (table 2). It may therefore be concluded that all the alcohols studied must be oxidized by ADH in the same manner as ethanol.

Ethanol, alone or together with small amounts of either n-propanol, isobutanol or isomyl alcohol, was given intraperitoneally to rats and the blood alcohol curves were estimated as shown in fig. 2. Table 1 shows that the oxidation rate of ethanol was slowed down by the presence of the other alcohols. From fig. 2 it is obvious that isobutanol was metabolized very slowly and was still present in low concentrations in the blood 7 hours after the administration. Thus isobutanol appears to be rather outstanding, in that it is oxidized very slowly and inhibits the oxidation rate of ethanol more than the other alcohols tested.

Tabel 1

Metabolism of ethanol in rat liver homogenate and in rat *in vivo* in the presence of various aliphatic alcohols. The ratio between the concentrations of ethanol and other alcohols is 100:1 in the liver homogenate experiment and 10:1 in the *in vivo* experiments.

Additions		µmoles of alcohol metabolized per hour per gram of liver			
		<i>In vivo</i> (liver homogenate)		<i>In vivo</i>	
		Ethanol	Other alcohols	Ethanol	Other alcohols
Ethanol	1	33		8.9	
	2.			8.8	
	3			7.6	
Ethanol + n-propanol	1	12	0.6	7.3	0.8
	2.			6.7	0.7
Ethanol + isobutanol	1	20	0.4	3.4	0.2
	~			4.3	0.2
Ethanol + iso myl alcohol	1	12	0.4	6.0	0.4
	2.			5.0	0.3

Tabel 2

Effect of pyrazole on the rate of metabolism of various alcohols in a rat liver homogenate.

Additions	µmoles of alcohol metabolized per hour per gram of liver			
	Ethanol	n-propanol	isobutanol	isoxyl alcohol
No pyrazole	33	40	38	25
Pyrazole (final conc. 1 mM)	13	15	13	11
Inhibition %	33.6	62.5	65.0	54.4

Unexpectedly and unaccountably ethanol administered alone gave a higher maximum blood ethanol concentration (1.14 mg/ml) than an equal amount of ethanol given together with the fusel alcohols (0.69–0.88 mg/ml). In this connection isobutanol also has a more pronounced effect than the other alcohols (fig. 2).

Oxidation of aliphatic aldehydes

To investigate the intracellular distribution of aldehyde dehydrogenase, acetaldehyde was incubated together with either a liver homogenate, a

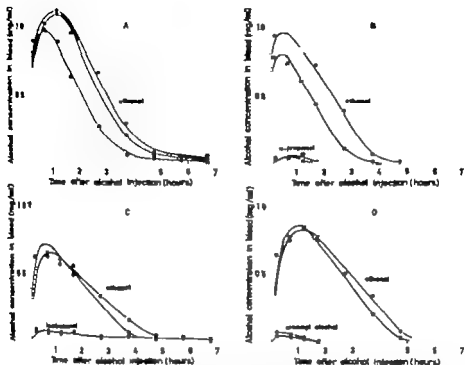


Fig. 2. Blood alcohol concentrations in rats given ethanol alone or together with small amounts of other alcohols. Ethanol alone (3.4 μ moles per 100 g body weight) or together with another alcohol (0.34 μ moles per 100 g body weight) was given intraperitoneally and tail blood was collected for gas chromatographic analysis. A. Ethanol alone. B. Ethanol together with *n*-propanol. C. Ethanol together with isobutanol. D. Ethanol together with lauryl alcohol. Fig. A represents three, fig. B, C and D two separate experiments.

supernatant free of mitochondria or the corresponding mitochondrial suspension. The rates of metabolism of acetaldehyde in the homogenate and in the mitochondrial suspension were rather high and of about the same magnitude (fig. 3). However when acetaldehyde was incubated with the supernatant free of mitochondria, the rate of metabolism was very low (fig. 3). By varying the concentrations of the different components of the incubation mixture (especially NAD and pyrazole), the low rate of metabolism was found not to be due to one or more components present in too low or toxic concentrations. Thus the results indicate that the aldehyde dehydrogenase (ALDH) is mainly found inside the mitochondria.

The oxidation rates of the aldehydes corresponding to the lower aliphatic alcohols studied, as described in the previous section, were

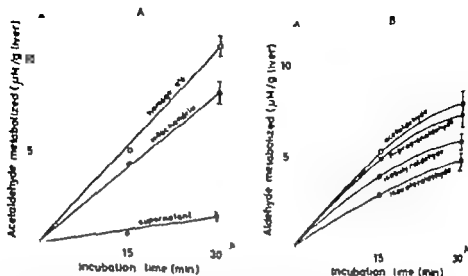


Fig. 3 Rate of metabolism of aliphatic aldehydes in homogenate, supernatant and isolated mitochondria from rat liver. A. 13.2 μmoles of acetaldehyde were incubated together with homogenate supernatant or mitochondria. Final incubation volume was 3 ml. B. 13.2 μmoles of aldehyde were incubated together with mitochondria. Final incubation volume was 3 ml. The aldehyde present at various incubation times was estimated by means of gas chromatography. The curves represent values of 6–10 experiments. The bars at 30 min. show the standard errors.

measured by incubating the aldehydes with mitochondria in the same way as described for acetaldehyde. From these experiments it is obvious that the rates of metabolism of the aldehydes become slower with increasing carbon-chain length (fig. 3). It should be noted that the aldehydes could not be reduced to the corresponding alcohols, since the enzyme (ADH) needed is extramitochondrially located (NYBERG, SCHUBERT & ÅNGOÅRD 1953). The most interesting finding is the slow rate at which isovaleraldehyde is oxidized compared with the other aldehydes.

If the oxidation rate was studied as oxygen consumption by the Warburg technique (table 3) or with the platinum micro-electrode, the same results were obtained as with the gas chromatographic method (fig. 3).

When acetaldehyde competed for the mitochondrial ALDH with another aliphatic aldehyde, the following results were obtained. The rates of metabolism of acetaldehyde and *n*-propionaldehyde when incubated together were about half those when each one was incubated alone. This was also the case with acetaldehyde and isobutyraldehyde. When acetaldehyde and isovaleraldehyde were incubated together the rates of metabolism became very slow compared with those obtained when each one was incubated alone. The results are presented in table 4.

Tabel 3

Rate of oxidation of aliphatic aldehydes in rat liver mitochondria. Oxygen uptake measured by Warburg technique. The figures are mean values of 2-5 experiments.

Additions	µatm. O ₂ consumed per hour per 10 mg prot.	
	Concentration of aldehyde 6.6 mM	Concentration of aldehyde 33.0 mM
Acetaldehyde	13.5 ± 1.7	24.6 ± 2.1
n-propionaldehyde	7.4 ± 0.6	21.3 ± 0.4
isobutyraldehyde	8.1 ± 1.1	18.9 ± 0.3
isovaleraldehyde	6.3 ± 0.8	8.1 ± 1.7

Tabel 4

Competition between acetaldehyde and other aliphatic aldehydes for ALDH in mitochondria. Concentration of each aldehyde in the incubation 4.4 mM.

Additions	µmoles aldehyde oxidized per hour per gram liver	
	The two aldehydes incubated separately	The two aldehydes incubated together (in equal amounts)
Acetaldehyde	14	5
n-propionaldehyde	10	4
Acetaldehyde	11	5
isobutyraldehyde	10	4
Acetaldehyde	11	2
isovaleraldehyde	5	

Aldehydes as inhibitors of mitochondrial respiration.

Aldehydes as inhibitors of the mitochondrial oxidation of various substrates were studied by the Warburg method and the platinum micro-electrode technique. Isovaleraldehyde was found to be the most potent inhibitor. From the results in table 5 and those in table 3 and fig. 3 one may conclude that this property of isovaleraldehyde depends on two facts: the mitochondria oxidize isovaleraldehyde more slowly than the other aldehydes and, at the same time, isovaleraldehyde momentarily inhibits the respiration more efficiently. Thus isovaleraldehyde is a stronger inhibitor of the oxidation of substrates in mitochondria than are the other aldehydes tested.

Table 5

Aldehydes as inhibitors of mitochondrial respiration. Substrate and aldehyde concentration in Warburg experiments 10 mM and 1.5 mM respectively and in oxygen graph experiments 10 mM and 1 mM respectively

Substrates	Estimated with Warburg technique				Estimated with platinum microelectrode			
	Acet aldehyde	n-propion- aldehyde	Isobuty- aldehyde	Isosol- aldehyd	Acet aldehyde	n-propion- aldehyde	Isobuty- aldehyde	iso ket aldehyde
α-glycerophosphat	0	10	15	50	0	0	0	11.2
Succinate.	0	0	0	0	2.7	9.8	19.2	19.6
β-OH butyrate	0	0	0	70	0	0	10.5	52.5
Glutamate.	0	5	11	40	30.3	41.2	28.4	62.6
Pyruvate-malate	18	21	0	38	45.0	41.3	50.3	69.0

~ Inhibition of respiration

Discussion

Oxidation of aliphatic alcohols

It has been shown by several authors (LUTWAK MANN 1938 THEORELL & BONNICHSEN 1951 WINER 1958 THEORELL 1965 VON WARTBURG, PAPPENBERG & AIBI 1965) that liver ADH from different species has a broad specificity to lower aliphatic alcohols. Furthermore THEORELL & YONETANI (1963) reported that liver ADH activity is specifically inhibited by pyrazole. This finding, together with our observation that pyrazole inhibits the oxidation of the fusel alcohols in a rat liver homogenate (table 2), confirms that the lower aliphatic alcohols investigated are oxidized by liver ADH.

NEYMARK (1938) and BERGGREN (1938) showed that in the dog and the rabbit the rates of metabolism of methanol, ethanol and propanol increased with increasing numbers of carbon atoms. THEORELL & BONNICHSEN (1951) and WINER (1958), using horse liver ADH, found that the initial oxidation velocities of *n*-propanol and isoamyl alcohol were faster than that of ethanol. On the other hand, LUTWAK MANN (1938) using horse liver ADH, and BARRON & LEVINE (1952), using yeast cell and yeast crystalline ADH have shown that ethanol is metabolized more rapidly than *n*-propanol, isobutanol and amyl alcohol. Our observations, that the oxidation rates of *n*-propanol and isobutanol are higher than that of ethanol in a rat liver suspension and in liver perfusion experiments (fig. 1), are in good agreement with NEYMARK's (1938) and BERGGREN's (1938) observations, whereas our conclusion that isoamyl alcohol, which is the main alcohol in fusel oil, is oxidized at a somewhat slower rate than ethanol, agrees with those of LUTWAK MANN (1938). This rather slow oxidation of isoamyl alcohol may contribute to an accumulation of metabolites, which we assume follows the consumption of certain alcoholic beverages. Thus an accumulation of the metabolite valeric acid may contribute to the hangover since this acid has been shown to cause a long-lasting depressive state in rats (HAGGARD *et al* 1945).

From table 1 it can be seen that the presence of a small amount of a lower aliphatic alcohol other than ethanol decreases the rate of metabolism of ethanol in a rat liver suspension as well as in the rat *in vitro*. These observations agree with the results by VON WARTBURG, RÖTHLISBERGER & EPPENBERGER (1961) where fusel oil was found to diminish the rate of metabolism of ethanol in the rat *in vitro*. Inversely with the exception of isobutanol the oxidation of these alcohols, even in low concentrations, is not prevented by the simultaneous oxidation of high concentrations of ethanol (table 1). This means that, of the fusel alcohols

studied, only isobutanol may accumulate in the body during the heavy drinking of certain alcoholic beverages, as for example when isobutanol is consumed simultaneously with large amounts of ethanol

Oxidation of aliphatic aldehydes

According to LUNDQVIST, FUCHMAN, KLÄNING & RASMUSSEN (1958), acetaldehyde is exclusively transformed into ethanol and acetate in a rat liver suspension under anaerobic conditions. Acetoin formation is of no quantitative importance (LUBIN & WESTERFELD 1945; LUNDQVIST *et al.* 1958).

RACKER (1949) has shown that not only acetaldehyde but also propionaldehyde, isobutyraldehyde and isovaleraldehyde are rapidly oxidized to the corresponding acids by a partly purified aldehyde dehydrogenase. GLENN & VANKO (1959) showed that liver slices, homogenates and rat liver mitochondria all oxidize acetaldehyde to acetate by means of the nonspecific NAD-requiring aldehyde dehydrogenase. Furthermore WALKENSTEIN & WEINHOUSE (1953) showed that the aldehydes mentioned above were all oxidized by rat liver mitochondria. They proposed that, of the wide variety of enzymes which have been reported to oxidize aldehydes, the mitochondrial enzyme appears to resemble most closely the dehydrogenase described by RACKER (1949). This suggestion, that aldehyde dehydrogenase is present in liver mitochondria, is supported by an investigation by MCQUIRE (1965). DIETRICH (1966) showed the aldehyde dehydrogenase to be active in the supernatant and in the mitochondrial fraction of rat liver and suggested that the enzymes in the supernatant and the mitochondrial fraction are distinct proteins. BÜTTNER (1965) on the other hand found that the aldehyde dehydrogenase was mainly located extramitochondrially. Thus so far the literature gives no final answer to the question of where in the cell the aldehyde dehydrogenase is concentrated. We were therefore prompted to contribute another investigation, the results of which will be seen in fig. 3. By inhibiting ADH with pyrazole the transformation of acetaldehyde into ethanol was omitted. As pyrazole does not inhibit mitochondrial oxidation (KJESSLING unpublished), the addition of pyrazole to a liver homogenate does not interfere with the oxidation rate of acetaldehyde either extra- or intramitochondrially. Our results, with only a slightly higher oxidation rate of acetaldehyde in the homogenate as compared with that in the corresponding amount of mitochondria, therefore indicate that most of the aldehyde dehydrogenase is located inside the mitochondria. WALKENSTEIN & WEINHOUSE (1953) determined the rates of metabolism

of acetaldehyde, n-propionaldehyde isobutyraldehyde, and isovaleraldehyde in mitochondria from rat liver as measured by the oxygen uptake in Warburg experiments. They found that the oxidation rates of the first three aldehydes were about the same and that the oxidation rate of the isovaleraldehyde was about half that of the others. RACKER (1949) added various aldehydes to the enzyme purified by his method and found that acetaldehyde was most rapidly oxidized. Our own results (fig. 3 and table 3), obtained by gas chromatography and by oxygen uptake by the Warburg technique and the platinum micro-electrode, are in good agreement with the observations of WALKENSTEIN & WEINHOUSE (1953) and of RACKER (1949). Particularly interesting is the remarkably slow oxidation of isovaleraldehyde. In another respect too isovaleraldehyde holds a unique position among the aldehydes studied. Table 4 shows that it reduces the rate at which acetaldehyde is oxidized. Thus isovaleraldehyde is itself oxidized comparatively slowly and at the same time causes a decrease in the oxidation rate of acetaldehyde, possibly by being a potent inhibitor of the electron transport system in the mitochondria.

Aldehydes as inhibitors of mitochondrial respiration

Aldehydes as inhibitors of mitochondrial oxidation have been described by REHAK & TRUITT (1958), BEER & QUASTEL (1958), KIESLING (1962a, b, c, & 1963), and MAJCHROWICZ (1965). KIESLING (1963) showed that the pyruvate oxidation in mitochondria from various tissues was initially strongly inhibited by acetaldehyde. He also found the prolonged inhibition of the oxidation of pyruvate in brain mitochondria to be due to the very slow oxidation of aldehyde in these mitochondria.

In the present paper these studies have been extended to include the effect of lower aliphatic aldehydes apart from acetaldehyde on the oxidation of some substrates in liver mitochondria. In the Warburg experiments the inhibition is low as the incubation time is 20 minutes, a time during which the aldehydes themselves are metabolized (table 3). This, in turn, causes a rapid decrease of the inhibition. In the oxygraph experiments, on the other hand, the inhibition is high as the registration occurs momentarily and no aldehyde is metabolized. From table 5 it can be seen that the most potent inhibitor even initially is isovaleraldehyde. As shown in fig. 3 this potent inhibitor is oxidized comparatively slowly in the body. These events may well contribute to the hangover i.e. by inhibiting the pyruvate oxidation in brain mitochondria.

Summary

1 This is the first paper of an investigation designed to increase our knowledge of the physiological mechanism involved in the phenomenon which follows heavy drinking and which is popularly called the "hang-over". Data are given about the oxidation rates of the fusel alcohols and their corresponding aldehydes in a rat liver homogenate, in rat liver *in situ* and *in vitro* and in isolated rat liver mitochondria. The oxidation rates of ethanol and acetaldehyde in the presence of the fusel alcohols or their aldehydes have been studied as well as the inhibitory effect of the aldehydes on the mitochondrial oxidation of various substrates. Finally the intracellular distribution of the aldehyde dehydrogenase has been estimated.

2. The oxidation rate of the alcohols is as follows

n-propanol > isobutanol > ethanol > isoamyl alcohol

3 The oxidation rate of ethanol *in vitro* in a liver homogenate and *in vivo* is decreased by the presence of small amounts of the fusel alcohols. Inversely the metabolism *in vivo* of isobutanol, even in low concentrations is markedly retarded by the simultaneous oxidation of ethanol.

4 The oxidation rate of the aldehydes corresponding to the previously mentioned alcohols is as follows: acetaldehyde > *n*-propionaldehyde > isobutyraldehyde > isovaleraldehyde. The oxidation rate of isovaleraldehyde is about half that of acetaldehyde.

5 The aldehyde dehydrogenase was found to be located mainly in the mitochondrial fraction of the rat liver. *n*-Propionaldehyde and isobutyraldehyde compete with acetaldehyde for the aldehyde dehydrogenase, whereas isovaleraldehyde markedly inhibits the acetaldehyde oxidation. Isovaleraldehyde is also the most potent inhibitor of the oxidation of various mitochondrial substrates.

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Central and Peripheral Catecholamine Turnover Studied by Means of ^3H DOPA and ^3H Tyrosine

By

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In a previous study (PERSSON & WALDECK 1968) the metabolism of catecholamines in the mouse brain and heart was investigated, using ^3H DOPA of a high specific activity. A complex time course of the ^3H -dopamine (DA) formed was observed. Furthermore, the early appearance of ^3H 3-O-methylamines suggested that newly formed catecholamines are preferentially released and metabolized. In the further analysis of these observations the experiments have been repeated in rats, where the brains can be more easily dissected into parts containing either DA or noradrenaline (NA) storing neurons. In addition, experiments using ^3H -tyrosine as precursor were included in the investigation. The high specific activities of the ^3H -precursors used allowed the use of lower doses than those employed in the majority of previous investigations.

Material and Methods

Male albino rats weighing 150-200 g were used. During the experiments they were kept at an environmental temperature of 30°. Specifically labelled H-L DOPA (ring 2,3,6- ^3H) with specific activity of 20 curies/mM or H-L-tyrosine (ring 3,5- ^3H) with a specific activity of 43 curies/mM both obtained from the Radiochemical Centre, Amersham, served as ^3H -catecholamine precursors. The precursors were given intravenously in doses of 2.5 and 5 $\mu\text{g}/\text{kg}$, respectively. After various time intervals the rats were killed with chloroform. The heart was removed, and the animal was exsanguinated. The head was separated from the body at the level of the foramen magnum. The spinal cord and brain were taken out and dissected free from associated blood vessels and meninges. The brain was dissected in the following way. After the lateral ventricles have been exposed by sectioning the corpus callosum and the fornix, the crus cerebri was taken out. The

remainder of the striatum was included in a part, called the hemispheres. To this part the cerebellum was added. The remainder of the brain, called the brain stem, consisted of the nucleus accumbens, septum, nucleus interstitialis striae terminalis (the tuberculum olfactorium followed the hemispheres) hypothalamus, thalamus and the remainder of the stem in the level of the foramen magnum. In some rats the levels of the endogenous catecholamines were determined separately. The levels of ^3H NA, ^3H DA, ^3H -normetanephrine (^3H NM) and ^3H -methoxytyramine (^3H MT) were determined as described elsewhere (PERSSON & WALDECK 1968). Carrier NA, DA, NM and MT (20 μg of each per sample) were added before the homogenization step. By fractionated elution of the Dowex column and determination of the radioactivity and native fluorescence of these extracts from rats injected with ^3H -tyrosine (for analytical details, see PERSSON & WALDECK 1968) it was established, that the ^3H -amines determined were not contaminated with other ^3H metabolites.

Results

Endogenous catecholamines in the different regions analysed for ^3H -catecholamines

After dissection as described above the NA and DA levels were determined in the hemispheres, brain stem caudate nucleus, spinal cord and heart (for definition of these regions, see above) according to standard methods. These results are presented in table 1.

^3H -amines after ^3H DOPA

Following the ^3H DOPA injection in the regions described above the ^3H DA, ^3H NA, ^3H MT and ^3H NM levels were determined at different

Table 1

Levels of endogenous catecholamines in the different dissected parts of the central nervous system. The method of dissection is described in Materials and methods. Shown are the mean \pm S.E.M. and within the brackets the number of determinations.

Tissue	Noradrenaline $\mu\text{g/g}$ tissue	Dopamine $\mu\text{g/g}$ tissue
Hemispheres	0.28 ± 0.02 (3)	0.37 ± 0.09 (3)
Brain stem	0.74 ± 0.03 (3)	0.38 ± 0.00 (2)
Caudate nuclei	0.34 ± 0.07 (3)	18.15 ± 2.44 (2)
Spinal cord	0.41 ± 0.07 (2)	0.12 ± 0.08 (2)
Heart	0.90 ± 0.11 (2)	0.03 ± 0.03 (2)

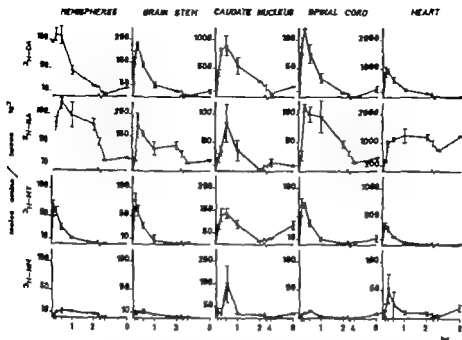


Fig. 1 Time-course of ^3H -noreadrenaline (NA), ^3H -dopamine (DA), ^3H -normetanephrine (NM) and ^3H -methoxytyramine (MT) in different regions of the central nervous system in the rat after the intravenous administration of $2.5 \mu\text{g/kg}$ ^3H -DOPA. The mean \pm S.E.M. of 3 experimental groups, each group consisting of 3 rats are shown. Note the different scales used for the ^3H -catecholamine laminates.

intervals. The time-course of the ^3H -catecholamines in the interval from $7\frac{1}{2}$ min. to 8 hrs is shown in fig. 1. The ^3H -catecholamine levels at the longer time intervals, i.e. at 16 and 41 hrs, are included in the semilogarithmic presentation in fig. 2, where some additional ^3H -catecholamine levels at 4 and 8 hrs are included. In fig. 3 the hemisphere levels are presented in the same way.

Within 15 to 30 min. after the ^3H -DOPA injection, the ^3H -DA and ^3H -MT levels reached a maximum in the different parts of the central nervous system. The ^3H -NA curves were perhaps slightly delayed in comparison with the ^3H -DA curves and reached a maximum within 30 to 60 min. In comparison with the ^3H -catecholamines, ^3H -NM occurred only in low amounts and showed little change. All the curves generally decreased to low values within 2 to 4 hrs after the injection.

In the heart the ^3H -DA and ^3H -MT curves had about the same shape as in the central nervous system. The ^3H -NA level decreased, however, at a slower rate than in the brain. The ^3H -NM curve did not follow that of its parent amine but had its maximum at about 15 to 30 min.

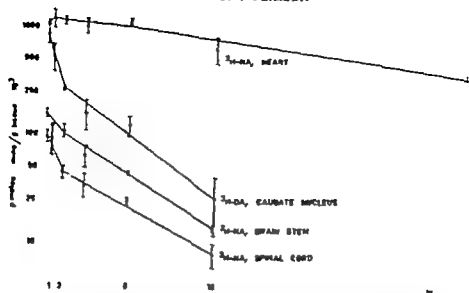


Fig. 2. Rate of decrease of ^3H -noradrenaline (NA) and ^3H -dopamine (DA) formed from ^3H DOPA (2.5 $\mu\text{g/kg}$ intravenously) in different regions of the central nervous system and the heart of the rat. The means \pm S.E.M. of 3-6 (at 4 hrs only 2) experimental groups, each consisting of 2 rats, are shown.

During the first 2 hrs after the ^3H DOPA injection considerable amounts of ^3H DA accumulated in regions which do not contain endogenous DA in appreciable amounts i.e. the spinal cord and heart. The maximal

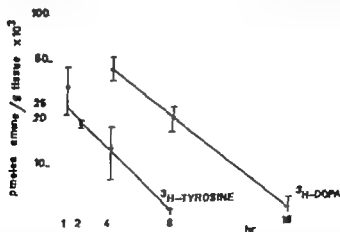


Fig. 3. Rate of decrease of ^3H -noradrenaline in the hemispheres (for definition of the parts see Materials and methods) of the rat with ^3H -tyrosine (5 $\mu\text{g/kg}$ intravenously) or ^3H DOPA (2.5 $\mu\text{g/kg}$ intravenously) as precursor. The means \pm S.E.M. of 3-6 experimental groups, each consisting of 2 rats, are shown.

^3H DA levels were about as high as the corresponding ^3H NA levels, except in the caudate nucleus, where they were about 10 times higher. After 2 hrs the distribution of ^3H DA seemed to be in agreement principally with that of endogenous DA.

The maximal ^3H NA accumulation was about the same in the hemispheres, spinal cord and caudate nucleus (about 0.1 pmoles/g.)¹⁾ In the brain stem the ^3H NA level was about twice as high. In the heart the maximal ^3H NA accumulation was about 1.0 pmoles/g.

The maximal ^3H DA accumulation in the caudate nucleus was about 1 pmoles/g., i.e. about 4 to 10 times higher than that of ^3H NA in the central nervous system. The corresponding ratio between endogenous DA and NA is considerably higher (table 1).

The apparent half-lives of the ^3H -catecholamines in the different regions were estimated by plotting the ^3H NA and ^3H DA levels in a semilogarithmic diagram (fig. 2 and 3). In the central nervous system, the half-life of ^3H NA seemed to be about 4–5½ hrs, shortest in the hemispheres, longer in the brain stem and longest in the spinal cord. These data are presented separately in table 2. In the heart the half-life of ^3H NA was much longer, i.e. about 21 hrs. The ^3H DA of the caudate nucleus seemed to have a half-life of about 4 hrs.

Table 2

Estimated half-lives of dopamine and noradrenaline in brain and noradrenaline in heart following administration of ^3H DOPA and ^3H tyrosine. The estimations were made from the data presented in fig. 2 and 3.

Tissue and H-amine	Half-life of the ^3H -amine when formed from	
	^3H DOPA hrs	^3H -tyrosine hrs
Hemispheres	4	3
^3H -NA		
Brain stem	4½	4½
^3H NA		
Spinal cord	5½	5½
^3H -NA		
Caudate nucleus	4	3½
^3H DA		
Heart	21½	23½
^3H -NA		

1) corresponding to 21 pg/g = 1 pg (picogram) = 10^{-12} gram.

³H-catecholamines after ³H-tyrosine

After ³H tyrosine (fig. 4) ³H DA* in the hemispheres, brain stem and caudate nucleus increased rapidly to a maximum at about $\frac{1}{2}$ -1 hr. In the spinal cord and heart the ³H DA levels were fairly low in contrast to the other regions. The ³H NA levels increased perhaps somewhat slower than ³H DA and the maximum seemed more extended in time. In the caudate nucleus the ³H NA accumulation was very low. In this region ³H MT increased rapidly to about 0.1 pmoles/g after 7 $\frac{1}{2}$ min. and then started to decline. In the other regions rather little ³H MT accumulated (about 0.01 pmoles/g). In contrast to the ³H DOPA experiments (fig. 1) the ³H NM curves showed well defined maxima at about 15 min. (0.02-0.04 pmoles/g).

After ³H-tyrosine the distributions of ³H DA and ³H NA were similar

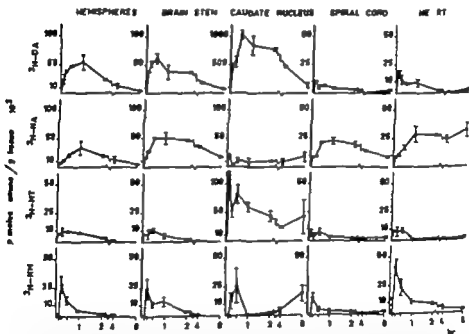


Fig. 4 Time-course of ³H-noradrenaline (NA), ³H-dopamine (DA), ³H-normetanephrine (NM) and ³H-methoxytyramine (MT) in different regions of the central nervous system in rat after the intravenous administration of 5 µg/kg ³H-tyrosine. The means \pm S.E.M. of 3 experimental groups, each group consisting of 2 rats, are shown. Note the different scales used for the ³H-catecholamines.

Although the specificity of the labelling is not 100% and the fate of the tritium in ring-3 position unknown, all data from the ³H-tyrosine experiments have been corrected for the loss of one atom of tritium per molecule.

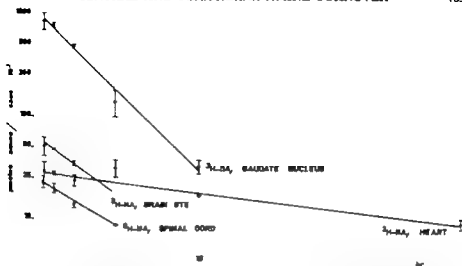


Fig. 5 Rate of decrease of ^3H -noradrenaline (NA) and ^3H -dopamine (DA) formed from ^3H -tyrosine ($5 \mu\text{g/kg}$ intravenously) in different regions of the central nervous system and the heart of the rat. The means \pm S.E.M. of 3-6 experimental groups, each consisting of 2 rats, are shown.

to those of endogenous DA and NA (compare fig. 4 and table 1) Low amounts of ^3H DA were thus found in the spinal cord and heart where there are no DA neurons and little or no endogenous DA occurs (see table 1)

The maximal ^3H NA levels in the hemispheres, spinal cord and heart were about 0.02-0.03 pmoles/g, in the brain stem about 0.05 pmoles/g and in the caudate nucleus about 0.01 pmoles/g. In the latter region the maximal ^3H DA accumulation was about 1 pmoles/g.

The apparent half-lives of the ^3H -catecholamines in the different regions were estimated in the same way as in the ^3H DOPA series (*vide supra*). The calculated half-lives after ^3H tyrosine were of the same order of magnitude as after ^3H DOPA though possibly somewhat shorter in the central nervous system (see fig. 3 and 5 and table 2) The half-lives of ^3H NA were thus in the hemispheres about 3 hrs, in the brain stem about 4½ hrs, in the spinal cord about 5 hrs and in the heart about 23 hrs. In the caudate nucleus ^3H DA had a half-life of about 3 hrs.

Discussion

In the present investigation the brains were dissected in such a way as to obtain "pure" samples, containing either endogenous DA or NA, i.e. the caudate nucleus and the spinal cord, respectively or mixed samples containing both catecholamines, i.e. the "hemispheres" and the brain

stem (cf table 1) In addition the heart, which contained NA but little or no DA was examined. This procedure allows of certain conclusions as to the fate of the administered precursors in the two types of neurons

Distribution of ^3H -catecholamines

The distribution of ^3H -catecholamines was not the same after the administration of the two ^3H -precursors. Following ^3H tyrosine the distributions of the ^3H -catecholamines were rather similar to those of the endogenous DA and NA respectively. This was not unexpected since tyrosine is probably the precursor supplied to the catecholamine storing neurons *via* the blood stream. The distribution of both endogenous and ^3H -catecholamines will then at least partly be determined by the activity of tyrosine hydroxylase, i.e. the rate-limiting step in catecholamine synthesis. However the distributions of endogenous and ^3H -catecholamines are not identical. For example, the ^3H NA of the heart was only half that of the brain stem in spite of a higher endogenous NA level, reflecting the lower turnover rate of NA in the peripheral than in the central neurons.

Following ^3H DOPA the distribution of ^3H -catecholamines was somewhat different. Particularly striking was the observation that now in contrast to the findings after ^3H tyrosine, large amounts of ^3H DA were found even in the "pure" NA regions, i.e. the spinal cord and the heart. The maximum ^3H DA levels were about the same as the maximum ^3H NA levels in these regions, but ^3H DA disappeared more rapidly as might be expected from a precursor. When evaluating the ^3H DOPA data it must be kept in mind, however that DOPA/5-HTP decarboxylase occurs not only in catecholamine neurons but also in 5-HT neurons (LOVÉNBERG, WEISSBACH & UDÉNFRID 1962) and extraneuronally e.g. in the capillary walls of the brain (BERTLER *et al* 1966, ÖWMAN & ROSEN-GREN 1967) and in the kidney. Extraneuronal ^3H DA accumulation is probably slight however owing to rapid deamination (BERTLER, FALCK & ROSENBERG 1963). This reservation probably applies only to ^3H DA and not to ^3H NA, since DA- β -hydroxylase activity probably occurs only in NA neurons (see JONASSEN & RUTLEDGE 1968).

The maximum yield of ^3H DA and ^3H NA in the different regions showed some interesting differences after administration of the two precursors. In the caudate nucleus the maximum yield of ^3H DA after ^3H DOPA was about twice that observed after ^3H -tyrosine. (When calculating the yield, the difference in precursor dosage was taken into account). In the spinal cord the maximum yield of ^3H -NA was 8 times

higher after the former than after the latter precursor. Consequently the DA neurons appear to utilize exogenous DOPA rather poorly. Whether the uptake or decarboxylation of DOPA constitutes the limiting factor in this respect, remains to be elucidated.

In the heart, the maximum yield of ^3H NA was about 80 times higher after ^3H DOPA than after ^3H -tyrosine, as compared to a factor of 8 in the spinal cord. Exogenous DOPA is thus utilized much more efficiently by the peripheral than by the central neurons. Two possible explanations of this difference may be considered, a) that DOPA does not penetrate easily through the blood-brain barrier and is partly lost through enzymatic degradation during this passage (cf BERTLER *et al* 1966) and b) that decarboxylation of exogenous DOPA may occur to a considerable extent extraneuronally e.g. in the kidney the DA thus formed may be taken up from the blood by the peripheral NA neurons but not by the central neurons, because DA does not readily pass through the blood-brain barrier.

^3H 3-O-methylamines

The accumulation of ^3H MT and ^3H NM depended to a considerable extent on the precursor used. Following ^3H -tyrosine, the distribution of ^3H MT was similar to that of ^3H DA, indicating that ^3H MT is mainly formed in DA neuroeffector units. ^3H NM was found in all the tissues examined. It is interesting to note that the peaks of the ^3H 3-O-methylated amines in general preceded those of the corresponding ^3H -catecholamines, indicating that newly formed catecholamines are preferentially metabolized. The magnitude of this early metabolism is difficult to estimate in view of the fact that the 3-O-methylamines in turn are probably rapidly metabolized. The 15-minute values for ^3H NM usually exceeded the simultaneous ^3H NA values, indicating that more than half of the ^3H NA formed initially undergoes rapid 3-O-methylation.

After ^3H DOPA the ^3H NM did not reach any high levels relative to ^3H -NA in the central NA regions. In contrast, ^3H MT was formed in appreciable amounts in both DA and NA regions. The reason for this difference between the two precursors with regard to the pattern of 3-O-methylated metabolites is not clear. Probably the higher ^3H MT values in NA regions following ^3H DOPA mainly reflect the higher ^3H DA values commented upon above. As to the high ^3H NM values (relative to ^3H NA) observed after ^3H -tyrosine but not after ^3H DOPA, it is tempting to speculate that they reflect a difference in the location of the ^3H NA formed from the two labelled precursors. Since O-methylation probably occurs only after release into the extraneuronal space (CARLSSON

& HILLARP 1962 KOPIN & GORDON 1962), preferential metabolism along this route would indicate the preferential formation of ^3H NA in a location readily available for release either because it is superficially situated within the nerve terminal or because it occurs in a labile, readily available pool. It is difficult to specify why the two precursors should be different in this respect. One possible explanation could be a different distribution of the enzymes involved e.g. tyrosine hydroxylase would tend to be more superficially located than DOPA decarboxylase.

In the study on catecholamine metabolism in mouse brain, using ^3H DOPA (PERSSON & WALDECK 1968) the time courses of the ^3H -catecholamine levels were markedly different from those observed in the present investigation in rats. The main difference was a more prolonged time course with later peaks and lower disappearance rates suggesting a slower ^3H precursor elimination. Further it could be seen that the ^3H NM peak clearly preceded the ^3H NA peak, indicating a preferential metabolism of newly formed ^3H NA. The present investigation indicates that this phenomenon is even more pronounced at least in the rat when ^3H -tyrosine is used as precursor.

Appreciable amounts of ^3H NM were found in the caudate nucleus after both precursors. This is possibly an analytical artifact related to the large amounts of ^3H DA present in these samples.

Apparent half-lives of ^3H -catecholamines

In spite of some scattering of the values obtained, the data allowed approximate estimations of the apparent half-lives of the ^3H -catecholamines in the different regions. Notwithstanding the differences in distribution of the ^3H -amines discussed above, the half-lives obtained were almost the same after ^3H DOPA as after ^3H tyrosine. The question arises whether these half-lives reflect the true turnover rates of the endogenous catecholamines. There are certain reasons to believe that this is not the case. Firstly the ^3H 3-O-methylamine data discussed above indicate that newly formed catecholamines are preferentially metabolized. This rapid initial phase of metabolism is not taken into account in the half-life estimation on the basis of ^3H -amine slopes. Secondly the half-life estimation is based on the as yet unproven assumption that ^3H precursors no longer are present during the period of examination. The remarkably long apparent half-life of the ^3H NA of the heart, as observed in the present investigation, i.e. more than 20 hours, calls for caution. Using ^3H NA, half-lives of about or less than 10 hours have been obtained in rat hearts (COSTA & NEFF 1966 IVERSEN, GLOWINSKI & AXELROD 1965 PICHLER,

SUKO & HERTTING 1968) * The reason for this discrepancy remains to be elucidated but persistent precursor labelling in the present experiment is a distinct possibility. Other possible explanations are differences in strain or experimental conditions. It should be noted that MONTAANU *et al* (1963), using labelled NA in a dose of 0.63 $\mu\text{g/kg}$ intravenously observed a slower rate of disappearance of the amine from heart than did the authors mentioned above.

Several previous authors have studied the catecholamine metabolism in the brain using labelled precursors (UDENFRIEND & ZALTZMAN-NIRENBERG 1963 BURACK & DRASKÓCZY 1964 GORDON *et al* 1966 DRASKÓCZY & LYMAN 1967 SEDVALL, WEISE & KOPIN 1968). In most cases their results are not strictly comparable with ours because of differences in dosage, route of administration, period of observation, species etc. However our conclusions concerning turnover rates are in rather close agreement with those of UDENFRIEND & ZALTZMAN-NIRENBERG (1963). In guinea pig brains they obtained half-lives of 4 and 3 hours for NA and DA respectively after ^{14}C tyrosine given intraperitoneally in a much higher dose than in the present investigation. The specific activity of free tyrosine was also measured and was found to be higher than that of the catecholamines throughout the experiments. It was concluded that one cannot really calculate turnover values for the catecholamines under such conditions. Following ^3H DOPA, again in a relatively large dose, similar half-lives were obtained. In a subsequent paper UDENFRIEND *et al* (1963) concluded that labelled DOPA is not suitable for the measurement of catecholamine biosynthesis, because it must be assumed to increase the pool size of endogenous DOPA. It seems doubtful however if this objection is valid for the ^3H DOPA of high specific activity available today. Experiments are in progress in which ^3H -precursor levels are followed in the blood plasma and various organs.

Attempts have been made to estimate turnover rates of catecholamines using synthesis inhibitors (IVERSEN & GLOWINSKI 1966 COSTA & NEFF 1966). The half-lives obtained in this way appear to be generally somewhat shorter than those of the present study. However the possibility that the inhibitors influence turnover rates, *via* depletion or by some other mechanism cannot yet be excluded. Another procedure which has been attempted, is to inject labelled catecholamines into the brain ventricles and to follow the rate of their disappearance from the brain tissue (IVERSEN & GLOWINSKI 1966). In this way half-lives of similar magnitude as with the other techniques have been obtained. The main shortcomings

* After an intravenous dose of 0.1 $\mu\text{g/kg}$ ^3H DL-norsadrenaline to rat half-life was estimated to about 14 hours (Persson & W. Ideck, unpublished).

of this procedure are that the cellular localization of the labelled amines is uncertain and that uptake occurs by a thin superficial layer of the brain, which may not be representative for the remainder of the brain tissue. In spite of these reservations the various procedures for investigating catecholamine turnover in the brain appear to be useful, particularly for comparative purposes, provided that due attention is paid to the sources of error. For example the differences in apparent turnover rates between different brain regions, as observed in the present study may very well reflect real differences in turnover rates. Similar observations have been made using histochemical techniques (Fuxe 1965), synthesis inhibitors and intraventricular injections of labelled catecholamines (IVERSEN & GLOWINSKI 1966).

Summary

Rats were injected intravenously with ^3H DOPA or ^3H tyrosine of high specific activity. At various intervals the animals were killed and the heart and different brain regions examined for tritiated dopamine, noradrenaline, 3-methoxytyramine and normetanephrine.

After ^3H -tyrosine the distribution of ^3H -catecholamines in different parts of the brain was similar to that of endogenous catecholamines. After ^3H DOPA the yield of ^3H -catecholamines was generally higher than after ^3H tyrosine the difference being most pronounced in the heart and smallest in the dopamine-rich region of the brain (the caudate nucleus). After ^3H DOPA in contrast to ^3H tyrosine relatively large amounts of ^3H dopamine were observed in dopamine-poor noradrenaline-rich regions.

The distribution of ^3H 3-methoxytyramine largely reflected that of ^3H -dopamine. ^3H normetanephrine was found in larger amounts (relative to noradrenaline) after ^3H -tyrosine than after ^3H DOPA, with maximum values distinctly preceding the ^3H noradrenaline peak values, indicating that newly synthesized ^3H noradrenaline is preferentially metabolized.

Apparent half lives of ^3H -catecholamines differed in different regions but were similar after the two precursors. Sources of error in turnover estimations by different techniques are discussed.

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Influence of Thyroid Function on the Acetaldehyde Level of Blood and Liver of Intact Rats during Ethanol Metabolism

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The establishment of a redox equilibrium between a substrate pair and the NAD-NADH couple of a cell compartment presupposes a sufficiently high activity of the dehydrogenase which catalyses the reaction. This qualification also holds for the redox equilibrium for the NAD-NADH₂ couple, and the substrate pair ethanol-acetaldehyde, in the yeast cell during both anaerobic and aerobic conditions (HOLZER *et al* 1946). It has been assumed that estimation of the redox state of the liver cytosol is possible by measuring the ethanol and acetaldehyde concentrations of this tissue (WOLF & KLEIN 1960). However the alcohol dehydrogenase reaction is the rate-limiting reaction for the degradation of ethanol, and consequently no redox equilibrium between these substrates and the liver cytosol is to be expected (BÜTTNER 1965). SCHOLZ (1967) found, nevertheless, that there is a good correlation between the redox equilibrium of the lactate-pyruvate and the ethanol-acetaldehyde substrate pairs in perfused rat liver.

Ethanol exerts a different effect on the redox state of the liver cytosol in hypothyroid and hyperthyroid rats (RAWAT & LUNDQVIST 1968 YLIKAHRI *et al* 1968). The work presented here was carried out with the aim of studying how treatment with triiodothyronine or propylthiouracil (lithyronium NFN) influences the acetaldehyde level, compared with changes in the redox state in the blood and the liver of intact rats during ethanol oxidation.

Material and Methods

Male Wistar rats, four months of age, and given ordinary laboratory food and tap water *ad libitum*, were used for the experiments. Hypothyroidism was induced by the daily

administration of 5 mg per 100 g body weight of a 0.5% solution of propylthiouracil (PTU obtained from Eli Lilly and Co., Indianapolis, USA) by stomach tube for three weeks. Hyperthyroidism was induced by daily treatment with 20 µg per 100 g body weight of 3,3',5-triiodo-L-thyronine (T_3 , Sigma Chemical Co., Missouri, USA) given intraperitoneally in saline, also for three weeks.

The oxygen consumption of the experimental animals in basal conditions was recorded at the end of the third week by means of a Beckman Oxygen Analyser Model E 2; the method used was that described by DEPOCAS & HART (1957). The basal oxygen consumption of the PTU-treated group was about 30 per cent lower than that of the control group and that of the T_3 -treated group about 30 per cent higher than that of the control group; this indicates that the animals developed pronounced hypo- and hyperthyroidism.

Fasting was effected by food depletion for twenty hours before testing. Ethanol, 1.5 g per kg of body weight, was given to all animals intraperitoneally as 10% (w/v) solution in saline. The animals were anaesthetised with 40 mg of pentobarbital (mebumalum NFN) per kg of body weight 15 minutes before sampling. Sixty minutes after the ethanol injection, blood samples were drawn from the tip of the tail, the abdomen was opened, a heparinised aspiration needle was inserted into the hepatic vein, and 2 ml of blood drawn into heparinised plastic catheter. The liver was then quickly frozen *in situ* with aluminium tongs precooled in liquid nitrogen. The frozen tissue was crushed in a mortar with the addition of liquid nitrogen and three parts of ice-cold 0.6 M perchloric acid. The precipitate was centrifuged down, and the supernatant was neutralised with 2 M K_2HPO_4 to pH 6.5–6.9.

Lactate and pyruvate concentrations were measured within a few hours of sampling by the enzymic method described by HOWARTH *et al.* (1959). Lactate dehydrogenase, NAD and NADH₂ were obtained from C. F. Boehringer (Mannheim, Germany).

Ethanol and acetaldehyde were measured in the acid sample by gas chromatography. The gas space technique was applied with Perkin-Elmer F 40 gas chromatograph. The temperature of the water bath used for the production of the gas space was 65°C. Tert-butanol was used as the internal standard. No corrections were made of acetaldehyde formation from pyruvate or from ethanol during the preparation of samples, since control experiments showed that only minor amounts were formed in an acid solution.

Results

During ethanol oxidation the levels of lactate and pyruvate and the lactate/pyruvate ratios in hepatic venous blood were generally in good agreement with the corresponding values in the liver (table 1). The lactate levels were markedly lower in fasted than in fed rats, both in the liver and in hepatic venous blood. Very low concentrations of lactate were found in the T_3 -treated rats after fasting. The pyruvate level was low in all groups.

In both fed and fasted rats given ethanol, the lactate/pyruvate concentration ratio was generally higher in those rats treated with PTU and lower in the rats treated with T_3 as compared with control rats (table 1). The lactate/pyruvate ratio was significantly higher in both liver ($P < 0.05$) and hepatic venous blood ($P < 0.01$) of the fed PTU-treated rats than in the fed controls. In the livers of fed T_3 -treated rats significantly lower ($P < 0.01$) ratios were found than in the control rats. The lactate/pyr

Table 1

Levels of lactate and pyruvate and lactate/pyruvate ratios after ethanol administration in liver and hepatic venous blood from normal, trichloro-thymine-treated and propylthiouracil-treated rats, fed and fasted, *in vivo*. One hour after administration of 2.5 g ethanol/kg body weight intra-peritoneally blood was drawn from the hepatic vein, and subsequently a sample of the liver was taken by freeze-clamp. The results indicate mean values \pm S.D. with the number of experiments in brackets.

	Lactate		Pyruvate		Lactate/pyruvate	
	hepatic venous blood nmol/ml blood	liver nmol/g wet wt.	hepatic venous blood nmol/ml blood	liver nmol/g wet wt.	hepatic venous blood	liver
Normal						
Fed	1190 \pm 100	1473 \pm 440	33 \pm 6	40 \pm 6	37 \pm 7	37 \pm 7
Fasted	910 \pm 160	1090 \pm 480	41 \pm 12	23 \pm 5	24 \pm 7	49 \pm 17
Trichloro-thymine-treated						
Fed	1670 \pm 300	1540 \pm 610	52 \pm 7	64 \pm 8	32 \pm 4	4 \pm 10
Fasted	300 \pm 120	260 \pm 140	37 \pm 9	45 \pm 13	8 \pm 3	6 \pm 3
Propylthiouracil-treated						
Fed	2350 \pm 320	2250 \pm 290	32 \pm 8	39 \pm 12	79 \pm 23	65 \pm 27
Fasted	870 \pm 160	690 \pm 410	20 \pm 7	21 \pm 9	51 \pm 23	32 \pm 12

ratios of fasted rats were much lower ($P < 0.001$) in T_3 -treated than in normal rats. As compared with the control rats, the lactate/pyruvate ratios of fasted PTU-treated rats were significantly higher ($P < 0.05$) in the blood, but slightly and not significantly lower in the liver.

The levels of ethanol in blood samples from the tail and from *vena hepatica* and in the intact liver tissue were determined 60 minutes after administration of ethanol. In both peripheral and hepatic venous blood (30–33 μ moles/ml blood) and in the liver (28–31 μ moles/g liver fresh weight) of all three groups of rats about the same levels were found.

Table 2 presents the levels of acetaldehyde found during ethanol oxidation. The concentration of acetaldehyde in the peripheral blood is significantly lower ($P < 0.05$) than in the hepatic venous blood or in the liver of all groups of rats. The concentration of acetaldehyde in hepatic venous blood was of the same order as, or usually slightly lower than that in the liver. The highest levels of acetaldehyde were found in normal rats. In each group of rats, the acetaldehyde level was lower when the animals had fasted than when they had been fed. The concentration of acetaldehyde in both the liver and hepatic venous blood of fed, PTU-treated rats, and in the liver of fed, T_3 -treated rats, was significantly lower ($P < 0.01$) than that in control rats. Fasted PTU-treated rats were found to have very low acetaldehyde levels in both hepatic venous blood and

Table 2

Levels of acetaldehyde in peripheral blood, hepatic venous blood and liver after ethanol administration to normal, triiodothyronine-treated and propylthiouracil-treated rats, fed and fasted. Ethanol was given, and samples were taken as described in the Methods section. Acetaldehyde was assayed by gas chromatography as also described in the Methods section. The results are given as mean values \pm S.D. with the number of experiments in brackets.

		Acetaldehyde concentration		Liver moles/g wet wt.
		peripheral blood μ moles/ml blood	hepatic venous blood μ moles/ml blood	
Normal				
Fed	(8)	16 \pm 8	100 \pm 44	135 \pm 22
Fasted	(6)		56 \pm 10	72 \pm 19
Triiodothyronine-treated				
Fed	(9)	24 \pm 8	70 \pm 79	83 \pm 31
Fasted	(6)		58 \pm 43	58 \pm 29
Propylthiouracil-treated				
Fed	(6)	23 \pm 11	41 \pm 13	65 \pm 15
Fasted	(4)		— \pm 1	21 \pm 2

liver and the differences were highly significant ($P < 0.001$) in comparison with the corresponding values in control rats. The acetaldehyde levels in the fasted T_3 treated rats were slightly lower in the liver but about the same in the hepatic venous blood as in the controls.

Discussion

The levels of the metabolites in the liver and in the hepatic venous blood are very close to each other (table 1 and 2), which is in close agreement with the observations made by SCHIMASSEK (1963) in liver perfusion experiments. The effect of the thyroid hormones on the redox state of the liver during ethanol oxidation has previously been studied by RAWAT & LUNDQUIST (1968) and by YLIKAHRI *et al.* (1968). RAWAT & LUNDQUIST were unable to demonstrate that the pretreatment of rats with thyroxine (levothyroxinum NFN) influenced the lactate/pyruvate ratio of liver slices during ethanol oxidation but observed that methylthiouracil and thyroidectomy increased the ratio. On the other hand YLIKAHRI *et al.* (1968) found that thyroxine treatment diminished the ethanol-induced rise of the redox level. The results presented in table 1 are with regard to thyroxine treatment, in accordance with those obtained by YLIKAHRI *et al.* (1968) and with regard to fed rats, support the observation made by RAWAT & LUNDQUIST (1968) in hypothyroid animals.

The low acetaldehyde concentration of the peripheral blood (table 2) may partly depend on extrahepatic aldehyde oxidation but also depends on the elimination of the expired air. The great difference between the acetaldehyde levels of peripheral blood and hepatic venous blood indicates the difficulty involved in drawing conclusions concerning hepatic acetaldehyde metabolism from analyses of peripheral blood. The extent to which acetaldehyde is evaporated from the lung is unknown. In every group of rats, the acetaldehyde level was lower if the animals had been fasted than if they had been fed.

The mid potential of the lactate-pyruvate redox pair is calculated to be -185 mv and that for the ethanol-acetaldehyde pair -197 mv (KREBS 1967). If a redox equilibrium was present between the two redox pairs, the ratio between the reduced and the oxidised partner of the pairs would be rather similar. However the values for the ethanol/acetaldehyde ratios are more than one order of magnitude higher than that for the lactate/pyruvate ratios. Furthermore, no correlation was discernible when the individual lactate/pyruvate ratios were plotted against the acetaldehyde levels of the treated or untreated animals.

The reasons for the differences in the acetaldehyde levels of the liver need to be found elsewhere than in the changes in the redox levels, at

least when larger amounts of ethanol are used. The acetaldehyde pool must be the resultant between the formation and the elimination of acetaldehyde.

It has been found that the capacity of intact liver tissue to oxidise ethanol is slower in both T_3 -treated and PTU treated rats than in control rats (HILLBOM, unpublished data). This finding confirms the results obtained by YLIKAHRI *et al* (1968) who found that slices from thyroxine treated rats oxidised ethanol at a slower rate than slices from normal rats. It has also been found, that liver slices from fasted rats oxidise ethanol at a slower rate than liver slices from fed rats (SMITH & NEWMAN 1959). Very little study has been devoted to the kinetics of acetaldehyde elimination in the intact liver during ethanol oxidation. It is known, however that the activity of the aldehyde dehydrogenase of rat liver is 4-5 times greater than that of the alcohol dehydrogenase (BÜTTNER 1965). Differences in alcohol dehydrogenase activity have been observed in livers from hypo- and hyperthyroid rats, but not between livers from fasted and fed rats (YLIKAHRI & MÄENPÄÄ 1968, HILLBOM & PIKKARAINEN 1968). The activity of the liver alcohol dehydrogenase is probably one factor which determines the rate of oxidation of ethanol to acetaldehyde. The acetaldehyde levels reported in table 2 and the activity of alcohol dehydrogenase in the differently treated rats quoted above indicate that no correlation exists between these two parameters. Whether differences exist in the aldehyde dehydrogenase activities of the livers of these animals is not known. If the acetaldehyde values presented here are compared with previous data concerning the ethanol oxidising capacity of liver tissue, it is apparent that the acetaldehyde level is lower in conditions in which the oxidation rate of ethanol is slower. For this reason, it is suggested that the acetaldehyde level might be related to the rate of ethanol oxidation.

Summary

The levels of acetaldehyde and the lactate/pyruvate ratio have been estimated in intact liver, hepatic venous blood and the peripheral blood of untreated, triiodothyronine-treated or propylthiouracil-treated rats after ethanol administration. The lactate/pyruvate ratio and the acetaldehyde levels were close to the values of the liver in hepatic venous blood, but the acetaldehyde levels found in the peripheral blood were low. The acetaldehyde levels were lower in starved than in fed rats, and lower in both hyperthyroid and hypothyroid rats than in normal animals. No correlation was found between the acetaldehyde levels and the redox state of the liver. A possible relation between the rate of alcohol oxidation and the acetaldehyde levels is discussed.

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From the Research Laboratories, AB Leo, Hålsjöborg, Sweden

Effects of Polymerized Oestrogens on the Ventral Prostate in Rats

By

Jonas Milstén

(Received May 7 1969)

Polyoestradiol phosphate, estradurin ® has been successfully used in the treatment of prostatic carcinoma (JONSSON *et al* 1963). Compared to treatment with diethylstilboestrol, which gave a marked inhibition of the release of the gonadotrophic hormones in human patients (BURKE *et al* 1954) polyoestradiol phosphate caused only a slight inhibition (GASTINEAU & ALBERT 1959 JONSSON *et al* 1963). Thus the effect of the latter compound is probably due to a direct action on the tumor cells in the prostate.

For this reason it seemed of interest to study the effect of polyoestradiol phosphate on the normal rat prostate. In addition to polyoestradiol phosphate two related compounds, polydiethylstilboestrol phosphate and polyoestriol phosphate, triodurin ® were included in the study.

Materials and Methods

The polymerized oestrogens were synthesized at the Research Laboratories, AB Leo Hålsjöborg, Sweden. They are water soluble compounds in which several oestrogen molecules have been united with phosphate bonds to form macromolecules (FERMÖ *et al* 1958). In the body oestrogen phosphate is slowly released from the polymer resulting in prolonged oestrogenic effect (DICEFALUS 1954 DICEFALUS *et al* 1956). In the tables polyoestradiol phosphate is called PEP, polydiethylstilboestrol phosphate PSP and polyoestriol phosphate SEP.

Antiandrogenic effect

Male rats (Wistar 200-350 g, maintained under standard conditions) were castrated and injected with testosterone-3-(p-hexyl-oxiphenyl)-propionate, andradurin ® 12 or 13 days after the operation. This testosterone ester has an even and prolonged androgenic effect, and causes marked growth of the degenerated prostate in castrated rats (Di-

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From the Research Laboratories, AB Leo Hålsjöborg, Sweden

Effects of Polymerized Oestrogens on the Ventral Prostate in Rats

By

Jonas Månitzing

(Received May 7 1969)

Polyoestradiol phosphate, estradurin ® has been successfully used in the treatment of prostatic carcinoma (JÖNSSON *et al* 1963). Compared to treatment with diethylstilboestrol, which gave a marked inhibition of the release of the gonadotrophic hormones in human patients (BIRKE *et al* 1954), polyoestradiol phosphate caused only a slight inhibition (GASTINEAU & ALBERT 1959 JÖNSSON *et al* 1963). Thus, the effect of the latter compound is probably due to a direct action on the tumor cells in the prostate.

For this reason it seemed of interest to study the effect of polyoestradiol phosphate on the normal rat prostate. In addition to polyoestradiol phosphate two related compounds, polydiethylstilboestrol phosphate and polyoestriol phosphate, triodurin ® were included in the study.

Materials and Methods

The polymerized oestrogens were synthesized at the Research Laboratories, AB Leo Hålsjöborg, Sweden. They are water soluble compounds which several oestrogen molecules have been linked with phosphate bonds to form macromolecules (FERMÉ *et al* 1958). In the body oestrogen phosphate is slowly released from the polymer resulting in prolonged oestrogenic effect (DICEFALUSI 1954, DICEFALUSI *et al* 1956). In the tables polyoestradiol phosphate is called PEP polydiethylstilboestrol phosphate DEP and polyoestriol phosphate SEP.

Anticarcinogenic effect

Male rats (Wistar 200-350 g, maintained under standard conditions) were castrated and injected with testosterone-3-(p-hexyl-oxiphenyl)-propionate, estradurin ®, 12 or 13 days after the operation. This testosterone ester has an even and prolonged androgenic effect, and causes marked growth of the degenerated prostate in castrated rat (DICK

FALUSY 1960) The three polymerized oestrogens were injected intramuscularly (0.5, 2 or 8 mg/kg) at the same time as the testosterone ester in order to interfere with this androgen-induced growth. Ten days later the rats were sacrificed and the ventral prostates were dissected free and weighed.

Antigonadotrophic effect

Male rats (Wistar 200-250 g, maintained under standard conditions) are castrated unilaterally and the removed testes were weighed. The remaining testis hypertrophies following an increased release of gonadotrophic hormones (cf. SMUTLEY 1962). In order to interfere with this increased release of gonadotrophic hormones, the three polymerized oestrogens were injected intramuscularly (0.5, 2 or 8 mg/kg) immediately after the operation. The injections were repeated ten days later to ensure a marked effect. After another ten days, the rats were sacrificed and the remaining testis and the ventral prostate were dissected free and weighed. The material for histological studies was fixed in Bouin, embedded in paraffin, sectioned and stained with eosin-hematoxylin.

Results

Antiandrogenic effect

The interference of the polymerized oestrogens in the androgen induced growth of the ventral prostates in castrated rats was weak (table 1). Only the highest dose of polyoestradiol phosphate and polydiethylstilboestrol phosphate significantly reduced the weight increase of the ventral prostates.

Antigonadotrophic effects

The effect of polyoestradiol phosphate and polydiethylstilboestrol phosphate on the weight of testes was very marked even with the lowest dose used but polyoestriol phosphate had a significant effect only with the highest dose (table 2). All three compounds caused varying degrees of atrophy of the ventral prostate indicating a diminished production of testosterone. Although the weight of the testes was not significantly altered after treatment with moderate doses of polyoestriol phosphate, the weight of the ventral prostate was significantly reduced.

Histologically no effect was observed in the testes after treatment with polyoestriol phosphate. The treatment with polyoestradiol phosphate and polydiethylstilboestrol phosphate caused a dose-dependent inhibition of spermatogenesis and a disorganization of the seminiferous epithelium. The lumen was reduced and partly filled with spermatocytes and multinuclear giant cells (fig. 1). In the ventral prostates the height of the epithelial cells was somewhat reduced after treatment with the highest dose of polyoestriol phosphate. The treatment with polyoestradiol

Table 1

The anti-androgenic effect of PEP, PSP and SEP as measured by the inhibition of the weight increase of the ventral prostate induced by the simultaneous administration of a testosterone ester in castrated rats.

Compounds	Dose mg/kg	n	Initial body weight g	Weight of ventral prostate mg	Change in body weight g
Andradurin.	30	18	212	103 ± 6	46
Saline	—				
Andradurin	30	9	11	97 ± 7	41
PEP	0.5				
Andradurin.	30	10	222	91 ± 5	24
PEP	2				
Andradurin.	30	10	214	88 ± 3 ¹	13
PEP	8				
Saline	—	10	214	12 ± 1 ²	9
PEP	8				
Andradurin.	30	9	333	190 ± 10	25
Saline.	—				
Andradurin.	30	5	355	154 ± 24	-13
PSP	0.5				
Andradurin	30	5	326	133 ± 27	-20
PSP	2				
Andradurin.	30	5	326	134 ± 6 ²	-22
PSP	8				
Saline.	—	5	343	46 ± 4 ²	-26
PSP	8				
Andradurin.	30	18	21	103 ± 6	46
Saline	—				
Andradurin	30	10	209	100 ± 9	19
SEP	0.5				
Andraduri	30	10	216	9 ± 12	37
SEP	2				
Andradurin	30	10	212	101 ± 7	40
SEP	8				
Saline	—	10	216	15 ± 8 ²	17
SEP	8				

¹ 0.01 > P > 0.001

² P < 0.001

phosphate and polydiethylstilboestrol phosphate caused a dose-dependent atrophy of the epithelial cells, a reduction of the lumen of the tubules and an increase of the fibromuscular stroma (fig. 2)

Table 2

The anti-gonadotrophic effect of PEP, PSP and SEP as measured by the inhibition of the compensatory weight increase of the remaining testes in hemi-castrated rats and the weight reduction of the ventral prostate.

Compound	Total dose mg/kg	n	Initial body weight g	Change in weight of testes mg	Weight of ventral prostate mg	Change in body weight g
Saline	-	40	209	278 ± 16	301 ± 13 (n = 20)	77
PEP	1	25	209	60 ± 23 ¹	94 ± 9 ¹ (n = 15)	40
PEP	4	25	203	-361 ± 52 ²	36 ± 3 ² (n = 15)	30
PEP	16	25	204	-521 ± 60 ²	30 ± 3 ² (n = 15)	19
Saline	-	20	219	52 ± 21	301 ± 13	73
PSP	1	20	17	-325 ± 69 ²	37 ± 5 ²	3
PSP	4	19	216	-677 ± 45 ²	25 ± 2 ²	-21
PSP	16	20	19	-586 ± 58 ²	29 ± 1 ²	-26
Saline	-	40	209	278 ± 16	301 ± 13 (n = 20)	77
SEP	1	25	207	239 ± 21	24 ± 15 ² (n = 15)	69
SEP	4	25	201	241 ± 24	40 ± 17 ¹ (n = 15)	66
SEP	16	25	200	194 ± 18 ²	175 ± 16 ² (n = 15)	51

¹ 0.01 > P > 0.001

² P < 0.001

Discussion

As reported by JÖNSSON *et al* (1963) polyoestradiol phosphate has a direct effect on human carcinomatous prostatic tissue. The results of the present study indicate that the direct effect on normal rat prostate is very weak. The antigonadotrophic effect of the compound, however, is of primary importance for the prostatic atrophy induced in rats. The primarily diminished release of gonadotrophic hormones causes a reduction in the release of testosterone resulting in an atrophy of the prostate. In this respect polyoestradiol phosphate and polydiethylstilboestrol phosphate had a marked effect in contrast to polyoestradiol phosphate. The effect of polyoestradiol phosphate and polydiethylstilboestrol phosphate can be compared to that of surgical castration.

The morphological changes in the testes and prostates after treatment with the polymerized oestrogens can be attributed to a diminished release of gonadotrophic hormones. Thus, marked changes in spermiogenesis were observed after treatment with polyoestradiol phosphate and polydiethylstilboestrol phosphate. The changes in the ventral prostates are probably due to a secondary reduction in the release of testosterone.

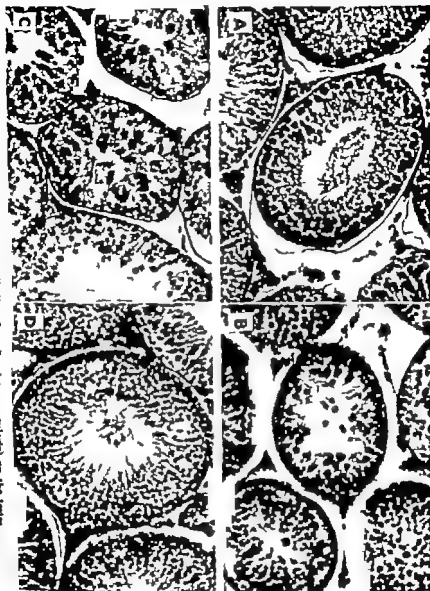


Fig. 1 The effect of PUP, PMP and BEP (2×8 mg/kg intramuscularly) on the testis.
 (A — saline, B — PUP, C — PMP, D — BEP. Magnification $150 \times$).

Polyoestrilol phosphate had such a weak antigonadotrophic effect that no changes in spermiogenesis could be detected. Nevertheless, the lowering of the secretory epithelium in the prostate indicates a diminished release of testosterone.

The results of this study compared to those of JÖNSSON *et al* (1963) illustrate the great differences between human carcinomatous prostatic tissue and the normal rat prostate.

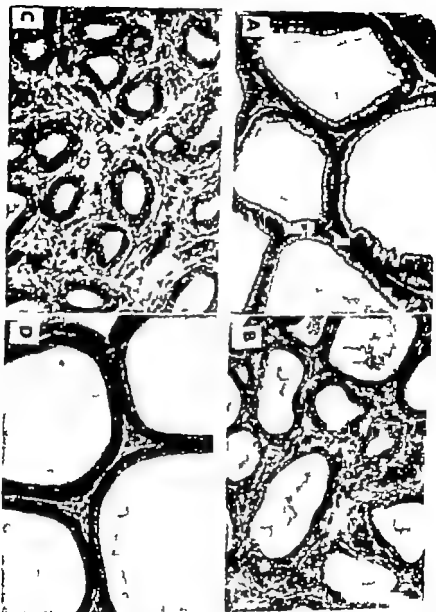


Fig. 2. The effect of PEP, PSP and SEP (2×8 mg/kg, intramuscularly) on the ventral prostate. (A = saline, B = PEP, C = PSP, D = SEP, Magnification 120 \times).

Summary

Three polymerized oestrogens – polyoestradiol phosphate, polydiethylstilboestrol phosphate and polyoestriol phosphate – were investigated to determine how these compounds influence the ventral prostate in rats. In moderate doses the compounds did not interfere directly with the androgen-induced growth of the ventral prostate. The morphological and

histological changes in the testes and ventral prostate indicated that the main effect was mediated by an inhibition of the release of gonadotrophic hormones and a reduction in the production of testosterone. Polydiethylstilboestrol phosphate and polyoestradiol phosphate had a high anti gonadotrophic effect, while the effect of polyoestriol phosphate was low.

Acknowledgement

The skilful technical assistance by Miss Inger Persson is gratefully acknowledged.

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From the Department of Pharmacology ■ J Medical College,
Poona, India

Effect of Zinc Sulphate on Carbon Tetrachloride Hepatotoxicity*

By

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(Received January 30, 1969)

Zinc metabolism is known to be affected in disorders of the liver. Liver cirrhosis in man is characterised by low serum and hepatic levels of zinc and elevated levels of zinc in the urine (VALLER *et al.* 1957). Corresponding findings have been reported with carbon tetrachloride induced liver cirrhosis in rats (KARN *et al.* 1968). It was, therefore, decided to study the effect of zinc on acute carbon tetrachloride induced liver damage in rats.

Materials and methods

Stock male albino rats, weighing 170-220 g. were used. Zinc sulphate ($ZnSO_4 \cdot 7H_2O$) was administered intraperitoneally as an aqueous solution in a dose of 2 mg/kg body wt. Five hours later carbon tetrachloride, 2.5 ml/kg mixed with an equal volume of liquid paraffin was administered subcutaneously. Control animals received physiological saline instead of zinc sulphate and only liquid paraffin instead of carbon tetrachloride, in corresponding amounts.

Liver function was studied 24 hours after the administration of carbon tetrachloride by hexobarbitone (enkephalium NFN) sleeping time, bromsulphalein (sulphobromophthalium NFN) excretion and hepatic succinate dehydrogenase activity using methods previously described by SRINIVASAN & BALWANT (1968).

A piece of liver was examined histologically for the extent of necrosis after staining with hematoxylin and eosin.

Statistical comparisons were made using Student's 't'-test for comparing means.

Results

From preliminary studies the dose of zinc sulphate was fixed at 2 mg/kg body weight. Simultaneous administration of zinc sulphate with carbon tetrachloride failed to show any protective effect.

Work presented at the XIVth Annual Conference of the Association of Physiologists and Pharmacologists of India, held at Baroda in December 1968.

Table 1

Hexobarbitone sleeping time.

Treatment	Number of animals	Sleeping time in min. \pm S.D.		P
		15 days before treatment	24 hours after treatment	
1 Blank control	13	18.3 \pm 2.1	17.5 \pm 2.4	1 V 2
2 Zinc sulphate	7	19.7 \pm 3.1	18.8 \pm 2.5	P > 0.1
3 Carbon tetrachloride	13	20.5 \pm 3.7	98.4 \pm 10.3	
4 Zinc sulphate + carbon tetrachloride	13	18.7 \pm 2.8	90.2 \pm 12.0	3 Vs 4 P > 0.1

The hexobarbitone sleeping time was prolonged after treatment with carbon tetrachloride. Pretreatment of the animals with zinc sulphate did not significantly reduce the sleeping time prolonged by the hepatotoxin (table 1).

Bromsulphthalein excretion was markedly impaired by carbon tetrachloride. Pretreatment with zinc sulphate significantly improved the ability of the liver to excrete the dye as evidenced by the serum bromsulphthalein levels (table 2).

Impairment of hepatic succinic dehydrogenase activity was less marked in the zinc sulphate-pretreated group than in the group treated with carbon tetrachloride alone (table 3).

Pretreatment with zinc sulphate, however did not reduce the extent of centrilobular necrosis and other histological changes produced by carbon tetrachloride. The extent of histological damage was found to be

Table 2

Bromsulphthalein excretion.

Treatment	Number of animals	Serum bromsulphthalein level (mg %) \pm S.D.	P
1 Blank control	13	1.32 \pm 0.11	1 Vs 2
2 Zinc sulphate	7	1.28 \pm 0.14	P > 0.1
3 Carbon tetrachloride	13	8.57 \pm 0.82	
4 Zinc sulphate + carbon tetrachloride	13	3.02 \pm 0.68	3 Vs 4 P < 0.001

Table 3

Succinic dehydrogenase activity of the liver

Treatment	Number of animals	Succinic dehydrogenase activity (μ g of triphenyltetrazolium chloride reduced/mg of liver tissue) \pm S.D.	P
1 Blank control	13	1.78 \pm 0.17	1 V 3
2 Zinc sulphate	7	1.87 \pm 0.18	P > 0.1
3 Carbon tetrachloride	13	0.85 \pm 0.09	
4 Zinc sulphate + carbon tetrachloride	13	1.43 \pm 0.20	3 V 4 P < 0.001

the same after carbon tetrachloride with or without pretreatment with zinc sulphate (fig. 1 and 2)

Discussion

Zinc plays many important roles in the normal physiology of plants and animals (VALLEE 1959). It is essential for the normal functioning of many enzymes and thus plays a definite role in intermediary metabolism (PRASAD 1967).

The metabolism of zinc has been shown to be altered in hepatic disorders. In human alcoholic cirrhosis of the liver serum and hepatic levels of zinc are reduced and the urinary excretion of the metal is increased. Such abnormalities are corrected by therapy with zinc sulphate, which also improves liver function as tested by bromsulphthalein excretion (VALLEE *et al* 1957).

Hyperzincuria and low levels of zinc in the serum and liver have also been reported in rats with experimentally induced cirrhosis induced by carbon tetrachloride (KAHN & OZIERAN 1967). The liver of such cirrhotic rats exhibits a higher uptake of an injected dose of Zn^{65} than normal rats (KAHN *et al* 1968). This means that zinc metabolism is involved in carbon tetrachloride induced liver damage. In acute carbon tetrachloride intoxication however Zn^{65} uptake by the blood, heart, kidneys and liver was not affected (BAXTER & SMITH 1962).

In the present study zinc sulphate produced significant improvement in the capacity of the liver acutely damaged by carbon tetrachloride to excrete bromsulphthalein. However the damage to the drug metabolising

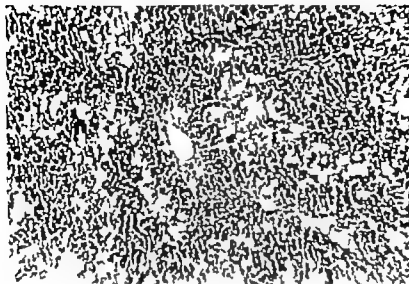


Fig. 1 Rat liver 24 hours after administration of carbon tetrachloride. Hematoxylin and eosin.

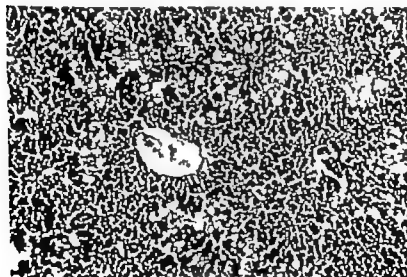


Fig. 2 Rat liver Pretreatment with zinc sulphate. 28 hours after the administration of carbon tetrachloride. Hematoxylin and eosin.

microsomal enzymes of the liver as tested by hexobarbitone sleeping time, was not altered. Nor was there any noticeable difference in the extent of histological damage.

The activity of hepatic succinic dehydrogenase, impaired by carbon tetrachloride, is improved by zinc sulphate. This may indicate a favourable effect of the salt on the mitochondrial damage produced by the hepatotoxin. Zinc is a constituent of many metalloenzymes, particularly the dehydrogenases, and in addition zinc increases the activity of many other enzymes, apparently as a cofactor in a nonspecific manner (PRASAD 1967). The effect of zinc sulphate on succinic dehydrogenase (an ion-sensitive enzyme) may thus be explained.

The long term effect of zinc on well established liver cirrhosis in rats (produced by hepatotoxic agents) is presently being studied in this laboratory.

Summary

Pretreatment of rats with zinc sulphate (2 mg/kg intraperitoneally) reduced the extent of liver damage produced by carbon tetrachloride (2.5 ml/kg subcutaneously administered 5 hours later) as studied by bromsulphthalein excretion and succinic dehydrogenase activity of the liver *in vitro*. The prolonged hexobarbitone sleeping time and the extent of histological damage of the liver were, however, not affected significantly by pretreatment with zinc sulphate.

Acknowledgements

The authors would like to thank Dr G. V. Joglekar for his helpful suggestions, Mr S. N. Deshpande for his help in the histological work and Dr S. S. Kekar and Dr Miss Usha Prabhu for their help in interpretation of liver histology and microphotography.

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From the Research Laboratories of Ferrosan Copenhagen, Denmark

Elimination and Distribution of Menbutone (Genabil®) in Rats

By

J. Lund and J. B. Lassen

(Received May 7 1969)

It has been established that menbutone (rINN), (genabil ® genabile ® genabile ® ido-genabil ® 3-(4-methoxynaphthoyl) propionic acid) has a stimulating effect on the secretion of bile and pancreatic juice (LASSEN & LUND 1967). Menbutone has been used in veterinary practice for the treatment of various digestive disorders (DAUWALDER 1963 FLÜCKIGER *et al* 1963 JOSEF 1965 MORRISON 1966 WINKLER 1967). In acute experiments on anaesthetized animals from which the bile and pancreatic juice were collected, the stimulating effect of menbutone lasted for 2-3 hours. The clinical effect after one treatment with menbutone, however seems to have a longer duration.

Determinations of menbutone-residues in tissues from pigs fed a menbutone-containing diet have been carried out. Pigs were given 10-12 mg/kg twice a day for 4 months (MADSEN & MORTENSEN 1966) and samples of the tissues were taken from the pigs after slaughter and analysed in this laboratory. In the tissues less than 0.2 p.p.m. menbutone were found in fat and muscle and less than 5 p.p.m. in the liver and kidney.

The purpose of this investigation was to study the elimination metabolism, enterohepatic circulation and the distribution of menbutone in rats.

Methods

1 Analytical Methods

¹⁴C-labelled menbutone was synthesized according to the scheme shown below¹⁾ (fig. 1).

The purity of the substance was tested by thin-layer chromatography (TLC) followed by autoradiography of the dried plate. The TLC was carried out in two separate systems.

¹⁾ The synthesis was performed by cand. pharm. J. A. Christensen, Ferrosan Ltd Copenhagen, Denmark.

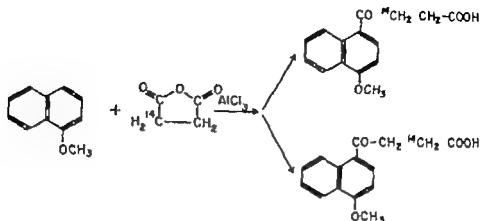


Fig. 1 Scheme for the synthesis of ^{14}C -labelled menbutone.

1) stationary phase Silica Gel III (Merck), mobile phase methanol, and 2) stationary phase Silica Gel III (Merck) mobile phase benzene, glacial acetic acid (9:1). In both systems only one radioactive spot was obtained. The specific activity was 8.5 $\mu\text{Ci}/\text{mg}$.

Determination of radioactivity

The radioactive material was extracted from the tissues by the following procedure. The entire organ or a sample of it was homogenized ("Ultra-Turrax") in about 10 vol. of 96% (v/v) ethanol. The homogenate was centrifuged for 15 min. at 4000 r.p.m. and the extract decanted. The residue was extracted with 2×10 vol. of 96% (v/v) ethanol and the combined extracts evaporated *in vacuo* ("Rotavapor"). This residue was dissolved in 2.00, 3.00 or 10.00 ml of 96% (v/v) ethanol. Of this extract 25 μl portions were used for measurements of radioactivity.

The extraction of fat was made in the same way but with chloroform instead of ethanol. The remainder of the carcass was ground in a meat grinder and representative 5.0 g sample was extracted with ethanol as described above.

Radioactivity in the extracts was determined on planchets (d = 3 cm) in a proportional counter (Fricke-Hoepfner FH 90), with automatic sample changer (F 516) and timer-printer (FH 449).

Two planchets were prepared from each extract and the results were corrected for self-absorption.

Samples of urine, blood and bile were pipetted (25 μl) directly onto planchets for measurements of radioactivity.

Menbutone added to urine, bile and organs gave 90–110% recovery when extracted and/or measured according to the procedures described above.

TLC-autoradiograph

50 μl of urine, bile or extract were developed on Silica Gel III with methanol and benzene, glacial acetic acid (9:1). ILFORD X RAY FILMS, ILFEX were exposed for 6 days for the autoradiographic examination.

Determination of menbutone in urine and bile

To 100–200 μl of urine or bile 10 ml of 1 M perchloric acid and 15.0 ml of chloroform

were added and the mixture was shaken for 20 min. A 10.0 ml aliquot of the chloroform phase was shaken together with 2.00 ml of 1 M borate (pH 10) for 20 min. To 1.00 ml borate phase was added 0.20 ml 0.5% sodium borohydride and the fluorescence of the corresponding alcohol was measured by fluorometry (Aminco-Bowman spectrofluorometer λ_{exc} 303 m μ , λ_{em} 371 m μ).

Menbutone concentrations were determined with the aid of standard curve.

Menbutone added to urine and bile gave 90–130% recovery when analysed according to this procedure.

2. Animal experiments

Excretion in urine and faeces and concentration in tissues

The labelled substance was added to non-radioactive menbutone and each animal was given 20 μ l (corresponding to 1.5 mg menbutone) in 4.0 ml 0.5% Na_2CO_3 . The solution was administered orally by stomach tube.

Two rats (2–3 months and 150 g) were used in each experiment. After the administration, the animals were placed in a cage with free water and food. Urine and faeces were collected separately in periods. In experiment 1 (exp. 1) 0–6 hrs and 6–18 hrs and in experiment 2 (exp. 2) 0–2 hrs, 2–4 hrs and 4–6 hrs. After 6 hours (exp. 2) or 24 hours (exp. 1) the animals were killed and the organs were removed for analysis.

Extrahepatic circulation

Two animals (210–225 g) were used in each experiment. The animals were anaesthetized with urethane (1.3 g/kg subcutaneously), and the trachea and vena jugularis were cannulated. The ductus choledochus was ligated about 0.5 cm from the liver hilus, and in order to collect the bile, a catheter was inserted into the ductus choledochus proximally to this ligature. The duodenum was ligated at the pylorus.

One hour fractions of bile were continuously collected. The labelled substance was added to non-radioactive menbutone (diethanolamine salt) to form a solution containing 2.0 μ l and 2.3 mg menbutone per ml and 1.0 ml of this solution was injected into the vena jugularis after the first hour of collection.

In the first of these experiments (exp. 3) the bile was collected. After measurement of the bile volume in the second of these experiments (exp. 4) the fractions were injected (except 25 μ l for analysis) through a cannula inserted into the duodenum.

Results

Excretion in urine and faeces and concentration in tissues

The excretion of radioactivity in urine and faeces after the oral administration of labelled menbutone is shown in table 1 and the cumulative excretion of radioactivity in the urine is shown in fig. 2.

In exp. 1 79% of the administered radioactivity was found in the urine 24 hours after the administration but only 4.4% of the dose was excreted in the faeces in the same period.

Within 6 hours after the administration 3% (exp. 1) and 2.3% (exp. 2)

Table 1

Excretion of radioactivity and menbutone in rats after oral administration of ^{14}C -menbutone (13 $\mu\text{Ci/kg}$, 10 mg/kg).

	Exp. 1 (24 hrs)		Exp. 2 (6 hrs)	
	% of dose ¹⁾ det. as menbutone	% of radioactive dose ¹⁾	% of dose ¹⁾ det. as menbutone	% of radioactive dose ¹⁾
Urine, 0-2 hrs	3	58	1	20
- 2-4 hrs			1	22
- 4-6 hrs			0.3	7.5
- 6-24 hrs			-	-
Faeces, 0-2 hrs	4	0.3	-	0.0
- 2-4 hrs			-	0.0
- 4-6 hrs			-	1.8
- 6-24 hrs			-	-
Total 0-6 hrs	-	58	-	51
excretion 0-4 hrs		83		-

1) Total doses exp. 1 1.5 mg, 2.11×10^6 cpm,
exp. 2 1.5 mg, 2.04×10^6 cpm.

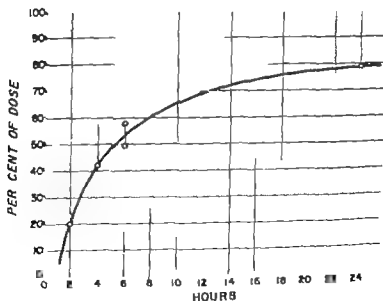


Fig. 2. Cumulative excretion of radioactivity in the urine from rats after oral administration of ^{14}C -menbutone (13 $\mu\text{Ci/kg}$, 10 mg/kg).

Table 2

Radioactivity in organs from rats 6 and 24 hours after oral administration of ^{14}C -menbutone (13 $\mu\text{Ci/kg}$, 10 mg/kg).

The amount of radioactivity in the organs are given for both animals (a and b) together with mean values.

	Animal	% of dose ¹⁾	
		Exp. 1 (24 hrs)	Exp. 2 (6 hrs)
Stomach incl. content ²⁾	{ a b }	{ 0.6 0.04 }	0.3
Stomach (wall)	{ a b }		{ 1.7 1.0 }
Gastric content	{ a b }		{ 6.0 3.1 }
Small intestine incl. content ²⁾	{ a b }	{ 0.3 0.2 }	0.3
Small intestine (wall)	{ a b }		{ 0.7 1.0 }
Content of small intestine	{ a b }		{ 1.1 2.8 }
Large intestine incl. content ²⁾	{ a b }	{ 1.8 1.5 }	1.7
Large intestine (wall)	{ a b }		{ 0.9 1.2 }
Content of large intestine	{ a b }		{ 6.1 12.5 }
Kidneys	{ a b }	{ 0.00 0.01 }	{ 0.01 0.1 }
Remainder of carcass	{ a b }	{ 0 0 }	{ 1.3 1.5 }

1) Total doses: Exp. 1 2.11×10^6 cpm, exp. 2 2.04×10^6 cpm.

2) The walls and the contents were not separated in exp. 1.

3) Including caecum and rectum.

were excreted in the urine as menbutone. The comparable figures for radioactivity were 58% (exp. 1) and 49.5% (exp. 2).

The radioactivity in the blood, brain, heart, lungs, liver, spleen, pancreas, fat and muscle was less than 0.2% of the administered dose in each organ. The activity in the kidneys, gastrointestinal tract and in the remainder of the carcass is given in table 2. As shown in table 2 the gastrointestinal tract was separated from its contents only in exp. 2. Six

hours after the administration (exp. 2) about 19% of the dose was present in the kidneys and the gastrointestinal tract, but 24 hours (exp. 1) after the administration only about 2% was found in these organs.

Menbutone could not be determined in the extracts from the tissues and faeces as the concentration was too low as compared with the blank values.

The total recovery of the administered radioactivity in the urine, faeces and tissues in exp. 1 was 86% and in exp. 2 73%.

TLC-autoradiography

Autoradlograms showed that only the faeces and urine extracts contained enough radioactivity to give visible spots.

Most of the radioactivity in the urine was found in a substance with an R_f -value different from that of menbutone. Developed with methanol, the radioactive substance had an R_f -value greater than that of menbutone, and developed with benzene-glacial acetic acid (9:1) the substance had an R_f -value smaller than that of menbutone.

This is in agreement with the results in table 1 and it is thus evident that most of the radioactivity in the urine is excreted in the form of a metabolite.

Radioactivity in the faeces was divided almost equally between a substance which behaved like menbutone on TLC plate and a substance with R_f -values almost the same as that present in the urine.

In exp. 2 the gastrointestinal tract was separated in "wall" and "content". The radioactivity in the content of the large intestine was divided between two substances as in the faeces.

Enterohepatic circulation

The excretion of radioactivity and menbutone in the bile of rats after intravenous administration of labelled menbutone is shown in table 3 and 4. In table 3 the results from exp. 3 are given. In this experiment a simple collection of the total amount of bile was performed. In table 4 the results of exp. 4 are given. Here the collected fractions of bile were injected into the duodenum after each collection period. The concentration of radioactivity in the bile is shown in fig. 3.

Table 3 and 4 and fig. 3 show that the total amount of radioactivity in the bile as well as the concentration is greater when the bile is injected. Thus, part of the radioactivity from the injected bile must have been absorbed from the intestinal tract and an enterohepatic circulation of menbutone must have occurred.

Table 3

Radioactivity and menbutone in the bile from rats after intravenous administration of ^{14}C menbutone (10 $\mu\text{Ci/kg}$, 11.5 mg/kg). The bile was collected continuously without injection into the duodenum (exp. 3).

Hours after administration	A (ml)	Volume in ml	Radioactivity % of dose ¹⁾	Menbutone % of dose ¹⁾	Radioactivity Menbutone
0-1	a	0.27	7.7	1.3	5.9
	b	0.46	16.1	11.6	1.4
1	a	0.35	6.6	1.0	6.6
	b	0.35	7.7	6.1	1.3
2-3	a	0.35	3.2	0.56	5.7
	b	0.35	3.4	3.4	1.0
3-4	a	0.40	1.15	0.20	5.8
	b	0.35	1.50	0.86	1.7
4-5	a	0.40	0.62	0.10	6.2
	b	0.40	0.84	0.39	2.2
5-6	a	0.42	0.30	0.05	6.0
	b	0.42	0.40	0.18	2.2

Total excretion 19.6%
 b 29.9%

¹⁾ Total doses 1.96 $\times 10^6$ cpm and 2.40 mg menbutone
 b 2.10 $\times 10^6$ cpm and 2.18 mg menbutone

The last column of table 3 gives the ratio $\frac{\text{radioactivity}}{\text{menbutone}}$ of dose of radioactivity / of dose of menbutone for each fraction. This ratio is rather constant during the experiment but there is a great difference between rat a and rat b.

The autoradiographic investigation of the fractions from exp. 3 rat a, showed that most of the radioactivity was found in a substance with an R_f -value different from that of menbutone. Developed with methanol on Kieselgel G 0.25 mm the radioactive substance had an R_f -value greater than that of menbutone, and developed with benzene, glacial acetic acid (9:1) the substance had an R_f -value smaller than that of menbutone. In the bile from exp. 3 rat b, most of the radioactivity was found in the menbutone spot. This is in agreement with the results in the last column of table 3.

Discussion

Absorption of ^{14}C menbutone from the gastrointestinal tract in rats is almost complete. Six hours after the oral administration only 16% is

Table 4

Radioactivity in the bile from rats after intravenous administration of ^{14}C -menbutone (10 $\mu\text{Ci/kg}$, 11.5 mg/kg). The bile was collected continuously in fractions and was injected into the duodenum after each period (exp. 4).

Hours after administration	Animal	Volume in ml	% of dose ¹⁾
0-1	c	0.40	11.5
1-2½	c	0.55	15.5
2½-3½	c	0.40	6.6
3½-4½	c	0.33	3.5
4½-5½	c	0.37	2.0
5½-6½	c	0.25	0.9
0-1	d	0.38	15.2
1	d	0.28	8.27
3	d	0.25	5.76
3-4	d	0.30	4.73
4-5	d	0.30	3.51
5-6	d	0.33	2.60
Total excretion c 40.0%			
d 39.1%			
) Total doses c 1.03×10^6 cpm			
d 2.03×10^6 cpm.			

found in the gastrointestinal tract and a part of this 16% may have been absorbed and excreted again with the bile.

By TLC and determination of menbutone in the urine it has been shown that most of the radioactivity in the urine has been excreted as one metabolite. This metabolite has not yet been identified. The same metabolite was found in the bile and faeces.

Deposition of menbutone or metabolites does not seem to occur in the rat, as the content of radioactivity in the animal 24 hours after administration was only 3%, which was found in the gastrointestinal tract. These results are in good agreement with previous investigations of tissues from pigs as mentioned above.

The difference from rat to rat of the ratio $\frac{\text{radioactivity}}{\text{menbutone}}$ in the bile may be due to an individual difference in the metabolism of menbutone in the liver.

In experimental animals from which the bile has been collected, one

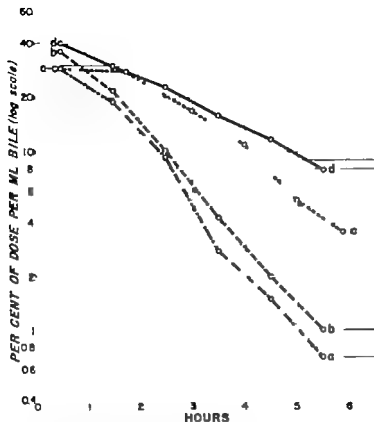


Fig. 3 Concentration of radioactivity in the bile of rats after intravenous administration of ^{14}C -menbutone (10 $\mu\text{Ci/kg}$, 11.5 mg/kg).

a and b Concentration without injection of the bile (exp. 3). and d: Concentration with injection of the bile into duodenum after each period of collection (exp. 4)

dose of menbutone has a choleretic effect of 2-3 hours duration. The clinical effect in pigs and cows of one dose is more prolonged. One possible reason for this discrepancy could be the enterohepatic circulation mentioned above.

Summary

The elimination and distribution of ^{14}C -menbutone after oral administration in rats were studied. The urinary excretion of the administered radioactivity was 50-58% in 6 hours and 83% in 24 hours. Most of the radioactivity in the urine was found in a metabolite not yet identified. About 4% of the radioactive dose was excreted in the feces in -

Determined as menbutone only 7% of the dose was found in the urine in 24 hours.

The residue of activity in the animal 24 hours after the administration was about 3 / and a considerable amount of this was found in the content of the gastrointestinal tract.

After intravenous injection of ^{14}C -menbutone into rats, 20-30 % of the radioactivity was excreted in the bile in 6 hours provided all the bile was collected. When most of the bile was injected into the duodenum both the total amount and the concentration of radioactivity in the bile were increased after each hour. This increased excretion after injection into the duodenum shows that there is an enterohepatic circulation of menbutone.

Acknowledgements

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Fatal Amitriptyline Poisoning Determination of the Drug in Forensic-Chemical Material

By

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Within the past 6-7 years amitriptyline (5-(3-dimethylaminopropylidene) 10 11-dihydro-5H-dibenzo [a, d] cycloheptene) has been extensively used as an antidepressive agent. Within this period five cases of fatal poisoning due to the drug have been seen in our laboratory. Several cases of this poisoning have been described previously (SUNSHINE & BLÜMLER 1963, SUNSHINE & YAFFE 1963, DAVIES & ALLAYE 1963, FORBES *et al* 1965), but the concentrations of the drug in the various organs have been determined in relatively few instances.

Method and Results

In order to establish an extraction procedure, the partition coefficients for amitriptyline between the organic phase (ether or chloroform) and aqueous phase were determined (table 1).

Table 1

Partition coefficients at room temperature for amitriptyline
between organic phase and aqueous phase.

Organic phase	Aqueous phase												
	0.1 M phosphate buffer pH						0.01	0.1	0.5	1	2	0.1	1
Ether	9.2	7.1	6.1	4.6	3.6	2.5	N	N	N	N	H	N	N
Chloroform	8.8	6.8	6.4	4.2	3.6	2.9	HCl	HCl	HCl	HCl	HCl	H ₂ SO ₄	H ₂ SO ₄
Ether	∞	∞	∞	0.56	0.13	0.07	0.03	0.06	0.03	0.06	0.04	0.06	0.06
Chloroform	∞	∞	∞	12.7	3.1	0.73	1.7	29	∞	∞	∞	0.03	0.13

This was done at room temperature using equal volumes of organic phase and aqueous phase with the addition of 10 μ g amitriptyline hydrochloride/ml buffer before the extraction. The extinction values of the buffer solutions in u.v. light at 238 m μ were measured before and after the extraction. Noteworthy are the partition coefficients between chloroform and hydrochloric acid. Evidently amitriptyline forms chloroform soluble complexes with chloride, similar to those found by KOTONS (1961) for the phenothiazines.

A. *Determination of amitriptyline in organs*

A portion of homogenized organ (without addition of water) was alkalinized by means of Na_2CO_3 (pH 9-10) and extracted three times, each time with three times its volume of ether the first time for 30 minutes. The combined ether extracts were then dried with anhydrous Na_2SO_4 , filtered, and evaporated in a rotation evaporator at 35-40. The residue was boiled down two or three times, each time with 10 ml of 0.1 N H_2SO_4 . The combined 0.1 N H_2SO_4 extracts were cooled and after readjusting the pH to one if necessary purified by extraction twice with equal volumes of ether. The 0.1 N H_2SO_4 extract was alkalinized (pH 9-10) and extracted three times each time with an equal volume of ether. The dried and filtered ether extracts were again evaporated in a rotation evaporator. The residue was dissolved in 5.0 ml of 0.1 N H_2SO_4 and its u.v. spectrum recorded.

B. *Identification of amitriptyline in the examined organs*

1. Spectrophotometric measurements of an aqueous solution of the drug gave the absorption curve characteristic of amitriptyline (and nortriptyline), with a maximum at 238 m μ and a minimum at 228 m μ .

2. Paper chromatography reversed phase according to STREET (1962), after spraying with iodoplatinate yielded spots with the same R_f value as amitriptyline. The liver also displayed spots with the same R_f value as nortriptyline. Elution with 0.2 N HCl of corresponding non-sprayed spots gave the same u.v. spectrum as amitriptyline and nortriptyline. It should be noted that the analyses were performed in the course of the past 5 years. To-day thin-layer chromatography is preferred.

C. *Recovery of amitriptyline from biological material in vitro*

Known amounts of amitriptyline hydrochloride (\sim three, ten and thirty μ g/ml) were added to 50 ml of whole blood and 50 g homogenized liver respectively obtained from a road casualty patient. The specimens

Table 2

Percentage of amitriptyline hydrochloride recovered from biological material *in vitro* using the method described under A.

	µg amitriptyline hydrochloride added		
	150	500	1500
50 ml blood	69	80	86
50 g liver	32	79	73

were left standing for about 30 minutes before being subjected to the stated extraction. The results are shown in table 2.

Case histories and findings

The five patients exposed to fatal amitriptyline poisoning were all women ranging in age from 22 to 45 years, who had been suffering from mental depression. Three of these women had been in mental hospitals because of this condition. The patients' ages, the amounts of amitriptyline taken, the intervals between the intake of the poison and death, and the concentrations of amitriptyline in the stomach contents, blood, and liver determined by the method described above, are shown in table 3.

Table 3

Concentrations of amitriptyline in different organs from five patients who had died of fatal poisoning by this drug. All the patients were women.

Case	Year of case	Age	Amount of amitriptyline taken	Interval between intake and death	Amounts of amitriptyline determined					
					Stomach contents		Blood	Liver		
					µg/ml	total in mg		µg/g	total in mg	of which nortriptyline
1	1963	27	ab. 5 g	ab. 7-8 hrs	1242	261	1	127	186	ab. 20
	1965	31	unknown	found dead but < 12 hrs	319	57	0	111	132	ab. 40
3	1965	32	unknown	found dead but < 24 hrs	3300	42	0	302	370	ab. 20
4	1965	45	unknown	ab. 36 hrs	4080	131	2	93	209	ab. 50%
5	1966	22	unknown	found dead	975	129		197	187	ab. 30%

1 weight not stated calculated from body weight.

Urine was only present in case no 2, where it was found to contain 0 µg/ml of amitriptyline, and in case no 5 where 8 µg/ml of amitriptyline was found, of which 20% was nortriptyline.

An acid-hydrolysis of the liver did not give higher values.

Other findings

In addition to the special analysis for amitriptyline the above cases were subjected to general forensic-chemical examinations. The results of these are shown in table 4

Discussion

The results of the chemical analyses have indicated that all the patients died following fatal poisoning after the oral intake of amitriptyline. The finding of empty saroten ® (amitriptylin NFN) bottles, in association with the mental condition of the deceased patients, afforded evidence suggestive of completed suicide in all the cases

The forensic-chemical analyses for amitriptyline showed no detectable amounts or a very low concentration in blood and high concentrations in stomach contents and liver

The low blood concentrations corresponded to those found by other workers (FORBES *et al* 1965 SUNSHINE & BAÜMLER 1963)

The concentrations in the stomach contents were very high as compared, for instance, with the 47 µg/g found by SUNSHINE & YAFFEE (1963) after

Table 4

Forensic-chemical analyses of the cases recorded in table 3
in addition to amitriptyline (except case 3 and 5)

Case	Drug	Stomach contents		Blood µg/ml	Liver	
		µg/ml	total in mg		µg/g	total in mg
1	thioridazine	329	69	0	6.5	9.6
2	probarbital	29	5.2	6	10	12
	(allypropymal NFN)					
	clonazepam	11	2	traces	0.6	0.7
4	pentobarbitone	traces		0	traces	
	(mebumal NFN)					
	phenytoin	traces		0	traces	

the intake of 1.25 g. However gastric lavage had been performed in this case, whereas this had not been done in any of the cases recorded in table 3. Four of the latter post mortem examinations revealed fragments of tablets in the stomach. SUNSHINE & BAÜMLER (1963) using STAS-Otto's method, likewise found a high concentration in the stomach contents (1000 µg/g).

The concentrations in the liver were high, but approximately in agreement with the amounts of non-converted drug (78 µg/g) found by SUNSHINE & YAFFEE (1963). Unfortunately however these workers did not report the analytical method used. But if the figures are compared with those obtained by FORBES *et al* (1965) and SUNSHINE & BAÜMLER (1963) using direct extraction, in the latter case combined with hydrolysis, their values are seen to be considerably lower (traces and 5 µg/g, respectively). This may be due to the analytical methods used, involving shaking from a chloroform to an acid aqueous phase, especially hydrochloric acid as used by FORBES *et al*. In this connection it must be admitted, however that FORBES *et al* (1965) found high concentrations (495 µg/g and 60 µg/ml, respectively) of non-converted amitriptyline in the urine without previous hydrolysis.

The figures set out in table 3 suggest a certain relationship between the proportion converted into nortriptyline in the liver and the interval between the intake of the poison and death. This is, however difficult to assess, because the time interval can be only roughly estimated.

Summary

Five cases of fatal amitriptyline poisoning have been subjected to forensic-chemical examination. The patients, all women ranging in age from 22 to 45 years, had been suffering from mental depression.

The concentrations of amitriptyline in the stomach contents, blood, and liver as well as in the urine when available, have been recorded, as has also the proportion of amitriptyline converted into nortriptyline in the liver.

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A Comparison of the Antibacterial Effect of Nitrofurantoin and Two Nitrofuran Compounds on Experimental *Escherichia Coli* Pyelonephritis

By

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(Received January 20, 1969)

Nitrofurantoin, a nitrofuran derivative, is a widely used antibacterial drug used for the treatment of urinary tract infections. It is characterized by rapid absorption and renal excretion, 20% by glomerular filtration and 80% by tubular secretion. A resorption in the distal part of the tubules has also been demonstrated both experimentally (BUZARD *et al* 1962 PAUL *et al* 1959 WOODRUFF *et al* 1961) and in man (SCHIRMEISTER *et al* 1966). Nitrofurantoin produces satisfactory antibacterial levels in the urine concomitantly with very low blood levels. In both dogs (KATZ & MOORE 1964) and man (COCKETT *et al* 1965) it has been found that the concentration of nitrofurantoin is higher in renal lymph than in blood.

In an autoradiographic investigation CURRIE *et al* (1966) have demonstrated the presence of nitrofurantoin in the renal interstitial tissue. The resorption in the distal tubules is partly dependant on the urine pH being markedly higher at low pH levels, when the antibacterial activity is also highest. This excretion-resorption mechanism is probably the explanation for the very good clinical results often found even with low doses of nitrofurantoin.

In experimental *Escherichia coli* (*E. coli*) pyelonephritis in rabbits and rats however we have found a less marked effect of nitrofurantoin than in experimental *E. coli* cystitis (AMAR 1961 PRÁTOR *et al* 1967 KONIČKOVÁ *et al* 1968).

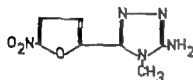
Serious side effects of nitrofurantoin are rare. Gastrointestinal side effects such as nausea and vomiting are present in 5-10% of cases, and

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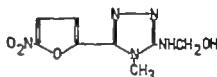
may sometimes prevent the use of the drug. This has prompted a search for analogues with less side effects and the same or higher antibacterial activity. In this communication the antibacterial effect of nitrofurantoin and two new nitrofuran compounds, Ph AA 65A and Ph AD 40A, on acute ascending *E. coli* pyelonephritis in rats is compared.

Ph AA 65A



3 Amino 4 methyl 5
(5-nitro 2 furyl)
1 2 4 triazole

Ph AD 40A



3 Hydroxymethylamino-4-methyl
5-(5 nitro 2 furyl) 1 2
4 triazole

Methods

The experiments were carried out on female albino Wistar rats weighing 180–200 g. An experimental model of acute ascending *E. coli* pyelonephritis was produced by a method similar to that described by HEDSTEDT (1964). A known amount of a nitrofurantoin-sensitive *E. coli* strain suspended in saline was injected into the urethra. In order to compare the effect of antibacterial drugs on renal infection in the intact and in the damaged kidney the left kidney was operated upon in all the animals before the injection of bacteria. The left ureter was ligated for 6 hours, the ligature was then removed and the bacterial suspension was injected 2 hours later. The details of this method were presented in an earlier paper (PRÁT *et al.* 1967). Therapy was started approximately 18 hours after the intra-urethral injection of bacteria. By this time, both kidneys were extensively infected as demonstrated in one special experimental group. Therapy lasted for seven days. Three to four days after terminating the therapy the rats were killed with ether. Under sterile conditions the abdominal cavity was opened and each kidney was removed for bacteriological examination. The kidney freed from fat tissue, was weighed and 0.5 g was homogenized, mixed with sterile saline and cultured in tenfold serial dilutions. After an overnight culture at 37°C on Endoplate, the number of colonies was calculated and multiplied by the given degree of dilution. The number of colonies was expressed per g of renal tissue.

The renal infection was produced with an *E. coli* strain (*E. coli* 5 7129). The serotype of this strain was O 129 O 133. This strain was sensitive *in vitro* to the antibacterial drugs under test. MIC (minimal inhibitory concentration) against nitrofurantoin being 6 µg/ml, against Ph AA 65A 4 µg/ml and against Ph AD 40A 8 µg/ml.

In each experimental group, half the number of animals was treated with one of these drugs and the other half was treated with a volume of 6% Macrodex corresponding to that of the drug suspension. All the drugs tested (nitrofurantoin, Ph AA 65A and Ph AD 40A) were suspended in 6% Macrodex. Each rat received, three times daily 20 mg of the drug/g body weight by stomach tube. This dose gives high levels in the urine but only low levels

Table 1

Urine and blood levels in 4 intact rats after a single dose of 20 mg/kg body weight per os.

	Urine			Blood	
	0-2 hrs	2-4 hrs	Total amount of drugs excreted! 4 hrs.	15 min.	40 min.
Furadantia	230 µg/ml	645 µg/ml	1.5 mg	-	-
	424 µg/ml	360 µg/ml	2.2 mg	-	-
Ph AA 65A	567 µg/ml	258 µg/ml	2.1 mg	4 µg/ml	3 µg/ml
	674 µg/ml	307 µg/ml	2.7 mg	4 µg/ml	-

In the plasma as can be seen from the polarographic analysis of the drugs in the urine and plasma (table 1). The quantitative bacteriological findings in both kidneys (the left kidney damaged by temporary ureteral ligature and the right intact kidney) of the treated rats and controls (treated with Macrodon) and the results in each of the treated groups (with nitrofurantoin, Ph AA 65A and Ph AD 40A) were compared.

Results

Bacteriological examination before start of therapy

A suspension of *E. coli* was injected into the urethra of 12 rats, after temporary ligature of the left ureter as described in Methods. Approximately 18 hours later the animals were killed and a bacteriological assay performed on each of their kidneys. A summary of the results is shown in fig. 1. With one exception, all the left damaged kidneys were heavily infected with colony counts greater than 10^5 /g. The right intact kidneys were, with one exception also infected, the colony counts being, however lower than in the left kidneys.

Bacteriological examination after therapy

The quantitative bacteriological findings in the renal tissue in the untreated controls and in the three treated groups are summarized in fig. 2. Therapy was started 18 hours after introducing the bacterial suspension. The kidneys of the animals treated with Ph AA 65A and Ph AD 40A were significantly less infected. In many cases, there was no bacterial growth. There were no significant differences between the rats treated with nitrofurantoin and the controls or between the three treated groups. In a small experimental group, therapy with nitrofurantoin and

L KIDNEY
(obstructed)

R KIDNEY
(Intact)

7

←● Mean
value

5

←● Mean
value

3

1

Log No of
bacteria g/tissue

Fig. 1 Quantitative bacteriological examination of kidneys 18 hours after intraurethral injection of a *E. coli* suspension into 12 rats. Before introducing the bacteria, the left kidney was damaged by unilateral ligature lasti g for 6 hours.

Ph AA 65A was started earlier than in the previous experiment. The bacteriological investigation showed that both drugs were more effective when therapy was started one hour after the intraurethral injection of bacteria (fig 3). Under these conditions, compound Ph AA 65A seemed to exhibit a higher antibacterial activity than nitrofurantoin and in all rats, the kidney tissue was free of infection. The effect of Ph AA 65A therapy is statistically significant as compared with the controls.

Discussion

The aim of this investigation was to compare two new nitrofurantoin compounds with nitrofurantoin. All three substances tested gave high urine levels. For testing the antibacterial effect *in vivo* a model for acute ascending *E. coli* pyelonephritis in rats was used. Therapy started relatively soon (18 hours) after introducing the bacteria into the urinary tract. The quantitative bacteriological investigation of renal tissue during this period showed that renal *E. coli* infection had become well established in most animals.

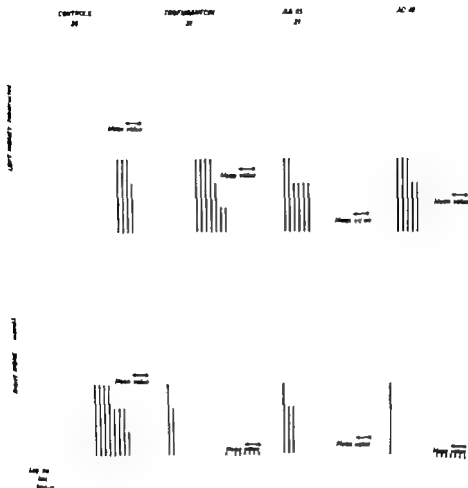


Fig. 2. Bacteriological examination after therapy which started 18 hours after the introduction of bacteria into the urinary tract.

Statistical analysis Chi-Square-test of the hypothesis that the frequency of significant positive bacteriological findings is the same in the four groups gives $\chi^2 = 17.55$ ($0.0005 < P < 0.001$) and the hypothesis is rejected. Simultaneous 95 per cent confidence intervals for contrasts (GOODMAN 1964) shows that there are significant differences between Ph AA 65A and the control and between Ph AD 40A and the control but no significant differences between Nitrofurantoin and the control or between nitrofurantoin and Ph AA 65A or Ph AD 40A.

Three days after termination of therapy which had been continued for 7 days, the quantitative bacteriological investigation of renal tissue revealed significant differences between the untreated controls and

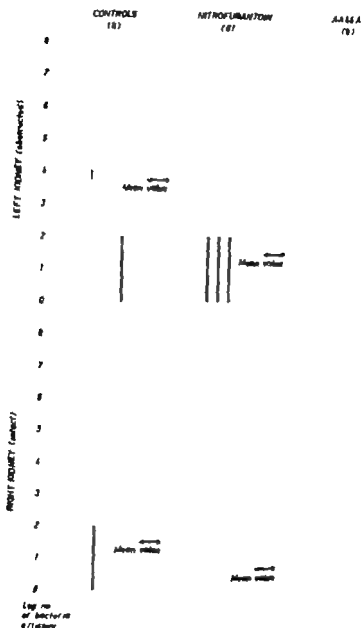


Fig. 3 Bacteriological examination after therapy which started 1 hour after the introduction of bacteria into the urinary tract.

The hypothesis that the probability of significant positive bacteriological findings is the same in the three groups is tested by paired comparison with Fisher's exact test. There is significant difference between the control and Ph AA 65 A ($P < 0.05$) but no significant difference between nitrofurantoin and the control or between nitrofurantoin and Ph AA 65 A.

groups treated with Ph AA 65A and Ph AD 40A. The kidneys of the treated rats were less infected and in several cases devoid of bacterial growth. The findings after treatment with Ph AA 65A and Ph AD 40A were more favourable than after treatment with nitrofurantoin.

The bacteriological examination in the experiment in which therapy was started one hour after introducing bacteria revealed a significant difference between Ph AA 65A and control but not between nitrofurantoin and Ph AA 65A or between nitrofurantoin and control.

From these results, it can be concluded that all three substances tested show antibacterial activity *in vivo* during the treatment of acute ascending *E. coli* renal infection. However bacterial growth was not absent in all the kidneys. These results do not unequivocally establish the antibacterial activity of the tested compounds during renal infection. Other studies under different experimental conditions, particularly in chronic bacterial infection should be done.

Summary

The antibacterial effect of nitrofurantoin and two new compounds (Ph AA 65A and Ph AD 40A) was tested on experimental acute ascending *E. coli* pyelonephritis in rats. The quantitative bacteriological investigation of renal tissue after therapy for seven days, which started 18 hours after the introduction of bacteria into the urinary tract, showed significantly better results in the rats treated with Ph AA 65A and Ph AD 40A than in the controls. There were no significant differences between nitrofurantoin and either of the new compounds or between nitrofurantoin and the controls. When nitrofurantoin or Ph AA 65A therapy was commenced one hour after the injection of bacteria into the urinary tract, Ph AA 65A seemed to be more effective than nitrofurantoin.

Zusammenfassung

Die antibakterielle Wirkung von Nitrofurantoin und zwei neuen Verbindungen, Ph AA 65A und Ph AD 40A, auf die experimentell erzeugte akute ascendierende Pyelonephritis von Ratten wurde untersucht. Die quantitative bakteriologische Untersuchung von Nierengewebe nach 7 Tagen Behandlung, 18 Stunden nach Einführung von Bakterien in die Urinwege begonnen zeigte im Vergleich zu Kontrollen statistisch gesicherte positive therapeutische Resultate bei Ratten die mit Ph AA 65A und Ph AD 40A behandelt worden waren. Keine statistisch gesicherten

Unterschiede zwischen Nitrofurantoin und den beiden neuen Verbindungen wurden gefunden. Wenn die Therapie mit Nitrofurantoin oder Ph AA 65A eine Stunde nach Einführung von Bakterien in die Harnwege begonnen wurde, scheint Ph AA 65A wirksamer als Nitrofurantoin zu sein.

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Effect of Different β -Adrenergic Receptor Blocking Agents on Hexobarbital Induced Narcosis in Mice

By

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Propranolol (proprasylyt NFN) has been shown to possess sedative and anticonvulsant properties which presumably are unrelated to its β -blocking activity (LESZKOWSKI & TARDOS 1965). Further evidence for the lack of correlation between central nervous depressant and β -adrenergic blocking activity was presented by MURMANN *et al.* (1966) who demonstrated that the β -blocking (–) isomer of 2-isopropylamino-1-(p-nitrophenyl)ethanol, HCl (INPEA) was not a CNS-depressant, while the (+) isomer which is devoid of β -adrenergic blocking properties, proved to be excitatory.

Studies by GRANA & SOSSI (1967) on the relationship between the CNS-depressant activity and the chemical structure of β -receptor blocking compounds led to the conclusion that the CNS-depressant properties are limited to the naphthyl- and naphthoxy-series.

The purpose of the present study has been twofold

- (1) to investigate whether the CNS-depressant properties are restricted to propranolol as a representative of the naphthoxy-series of β -adrenergic blocking compounds as compared with five β -blocking agents with ring structures other than propranolol, and
- (2) to elucidate the relationship between CNS-depression and β -adrenergic blockade by comparing the barbiturate-potentiating effect of propranolol with that of its two optical isomers, the (–) isomer possessing less than one hundredth the β -blocking effect of the (+) isomer (BARRETT & CULLUM 1968).

Material and Methods

Test drugs

The following compounds were used: 1-(isopropylamino)-3-(o-allylphenoxy)-2-propanol, HCl (II 54/22 – ptile ®), dachloroisoprenaline, HCl (DCI), 2-(isopropylamino)-1-(p-

nitrophenyl)ethanol, HCl (INPEA) 4-(2-isopropylamino-1-hydroxyethyl)urethane sulfoxanilide, HCl (MJ 1999) 1-(isopropylamino)-3-(*o*-phenoxyphenoxy)-2-propanol, HCl (Ph QA 33) propranolol (inalderal ®) and the (+) and (-) isomers of the latter compound.

All compounds were used as the hydrochloride corresponding to 86-88% of the base and were administered dissolved in saline. The doses mentioned refer to the salts.

Effect on hexobarbital sleeping time

The CNS-depressing activity of the test drugs was measured by their ability to prolong the hexobarbital (enhexymalum NFN) induced sleeping time in mice. Ten male NMRI mice born under specific pathogen free conditions, but kept under conventional conditions were used in each test group, while the control groups consisted of two groups, each of ten mice. The sleeping time of the control groups was determined in the morning and in the afternoon of the test day. The experiments were performed in the room in which the mice were kept: a temperature of 18-23° and a relative humidity of 50-60%. The sleeping time was defined as the time interval in minutes starting at the point where the mice could be placed on their back and lasting till their righting reflex was regained. The results are shown in table 1 and 2 as the mean \pm S.E.M. The influence of the test drugs on the sleeping time was determined by calculating the percentage deviation from the control value obtained on the same day. The relative potencies were expressed as the ED100 e.g. the dose causing 100% increase of the sleeping time. The ED100 values needed for qualitative comparison were derived from a semi-logarithmic plot of the observed sleeping times and are shown in column 6 of table 1 and 2. The test drugs were given intraperitoneally 30 min. before sleep was induced by 75 mg/kg hexobarbital Na given by the same route.

Determination of acute intraperitoneal toxicity (LD50 intraperitoneally)

In order to get a rough impression of the specificity of the CNS depressant activity the LD50 was determined according to the method of LITCHFIELD & WILCOXON (1949). Groups of five mice were used at each dose level and the percentage mortality recorded after one week.

Results

The effect of the different β -adrenergic blocking agents on the hexobarbital induced sleep is shown in table 1 and 2. Column 4 in the tables shows that the sleeping times of the control groups were very constant with a variation from 9 ± 1 to 12 ± 1 min. (mean \pm S.E.M., $n = 20$).

H 56/28 Ph QA 33 and propranolol caused a dose-dependent increase of the sleeping time the ED100's were of the same order of magnitude, the values being 16, 17 and 18 mg/kg respectively. When compared with the toxicity of the compounds, the ED100 values represent approximately 15% of the LD50. At the 10 mg/kg level both H 56/28 and propranolol showed a 40% increase of the sleeping time. At the 50 mg/kg dose level H 56/28 caused a fivefold increase while Ph QA 33 and propranolol increased it three to four times. When the toxicity of the compounds are considered, the CNS-depressant effect at the 50 mg/kg level may however be regarded as rather unspecific.

Table 1

Effect of H 56/28, Ph QA 33, INPEA MJ 1999 and DCI on hexobarbital induced narcosis.

Compound	Dose mg/kg L.p.	Sleeping time min. ¹ test	control	% Pro- longa- tion	ED100 ² mg/kg L.p.	LD50 ³ mg/kg L.p.
H 56/28	10	14 \pm 2	18 \pm 0.4	40	16	103 (81 131)
	20	20 \pm 3	11 \pm 1	82		
	30	30 \pm 1	10 \pm 0.4	200		
	50	53 \pm 4	11 \pm 0.4	381		
Ph QA 33	10	9.5 \pm 0.9	10 \pm 1	8	17	110 (92 121)
	20	19 \pm 2	9 \pm 1	111		
	30	29 \pm 3	10 \pm 0.4	190		
	50	36 \pm 4	10 \pm 0.3	260		
INPEA	30	10 \pm 1	10 \pm 1	0	190	205 (188 223)
	100	14 \pm 2	10 \pm 0.3	40		
	200	toxic	9 \pm 1			
MJ 1999	30	11 \pm 1	9 \pm 1	22	190	796 (771 916)
	100	13 \pm 1	9 \pm 1	67		
	200	18 \pm 3	9 \pm 1	100		
	300	27 \pm 5	12 \pm 1	123		
DCI	3	11 \pm 1	11 \pm 0.4	0	340	110 (77 121)
	10	30 \pm 4	11 \pm 1	150		
	30	17 \pm 2	12 \pm 1	42		
	50	7 \pm 2	11 \pm 0.4	-37		

¹ Mean \pm S.E.M

² Defined as the dose causing 100% increase of the sleeping time

³ Figures in brackets are the 95% confidence limits.

INPEA was tested at three dose levels including the toxic dose of 200 mg/kg. None of the dose levels had any significant influence on the sleeping time.

MJ 1999 showed an ED100 of 190 mg/kg, while 166 mg/kg was necessary to cause a significant prolongation of the sleeping time (67 increase). Thus, the CNS-depressing effect of MJ 1999 is considerably smaller than that of H 56/28, Ph QA 33 and propranolol. The toxicity of MJ 1999 is, however, also proportionally lower than that of the former compounds so that the relation between narcosis potentiation and the toxic dose level is almost the same for all four compounds.

DCI showed a biphasic dose-response relationship. At 3 mg/kg it was inactive. At 10 mg/kg it caused a 150% prolongation of the sleeping time, the effect of which was diminished at 30 mg/kg (42% increase) and

Table 2

Effect of propranolol and its optical isomers
on hexobarbital induced narcosis.

Compound	Dose mg/kg i.p.	Sleeping time min. ¹		/ Pro- longa- tion	ED100 ² mg/kg i.p.	LD ₅₀ mg/kg i.p.
		test	control			
Propranolol	10	14 ± 1	10 ± 1	40	18	135 (114-159)
	20	23 ± 3	10 ± 0.3	130		
	30	4 ± 4	10 ± 1	140		
	50	40 ± 3	11 ± 1	64		
(+) -Propranolol	3	13 ± 2	11 ± 1	18	16	
	10	21 ± 3	12 ± 1	75		
	20	21 ± 2	11 ± 1	91		
	30	29 ± 3	11 ± 1	164		
	50	41 ± 8	10 ± 0.4	310		
(-) -Propranolol	10	13 ± 2	1 ± 1	8	18	
	15	16 ± 1	10 ± 0.4	60		
	20	25 ± 3	11 ± 1	127		
	30	25 ± 3	11 ± 1	173		
	50	44 ± 2	12 ± 1	266		

¹ Mean ± S.E.M

² Defined as the dose causing a 100% increase of the sleeping time.

³ Figures in brackets are the 95% confidence limits.

reversed to a shortening of the sleeping time at 50 mg/kg. The latter is in accordance with an observed increase of the spontaneous activity of the mice at higher dose levels.

Table 2 shows a comparison of the barbiturate potentiating effect of propranolol as compared to its two optical isomers. All three compounds caused a dose-dependent increase of the sleeping time at the dose range 10-50 mg/kg, giving ED100 values between 16 and 18 mg/kg. The barbiturate potentiating effect of propranolol was thus indistinguishable from its (+) and (-) isomers.

Potential of barbiturate induced sleep may be due to factors other than CNS depressant activity. If the compounds to be tested induce hypothermia or in some way inhibit the enzymatic break-down of the barbiturate it would cause a prolongation of sleep. To exclude this possibility the β -blocking agents in some experiments were given intravenously immediately after the control mice had awoken from their sleep. It was shown that doses of 5-10 mg/kg H 56/28 Ph QA 33 and propranolol intravenously at this stage were capable of reinducing sleep in the mice.

Thus the compounds caused a potentiation of the subthreshold concentration of the barbiturate. This action is unlikely to be due either to a hypothermic effect or to an enzymatic inhibition. Therefore the prolongation of hexobarbital sleep described in this paper can be regarded as being due to a pure depressant effect on the central nervous system.

Discussion

GRANA & SOSSI (1967) have made a thorough study of the relationship between chemical structure and the influence on the CNS of β -adrenergic blocking agents. They concluded that compounds of the phenylethanol series (DCI, INPEA and MJ 1999) were either inactive or CNS-stimulants, while the naphthylethanol- and naphthoxypropanol amines (pronethalol (nafsylt NFN), propranolol) showed a depressant activity. The results presented here demonstrate that H 56/28, Ph QA 33 as well as propranolol cause a clear dose-dependent increase of the hexobarbital induced sleeping time of a similar order of magnitude. The CNS depressant effect observed in this study is therefore not restricted to the naphthoxy moiety present in propranolol, since H 56/28 and Ph QA 33 represent structures in which the bicyclic ring has been replaced by the allylphenoxy and phenoxyphenoxy groups (fig. 1). The finding that DCI caused a definite prolongation of the hexobarbital induced sleeping time at the dose range of 10–30 mg/kg was surprising, especially in view of the previously published data which indicate a CNS-stimulant action at the same dose range. MENNEAR & RUDZIK (1965) found that DCI potentiated the toxicity of d-amphetamine in aggregated mice at a dose of 10 mg/kg. Our observation that INPEA was inactive at all dose levels is in accordance with the results obtained by MURMAN *et al.* (1966). MJ 1999 represents another exception from the previously mentioned observation by GRANA & SOSSI that phenyl ethanolamines in general were either inactive or CNS-stimulants. This compound which has a methanesulfonanilide group attached to the phenyl ring revealed a significant barbiturate potentiating effect at 100 mg/kg. Although the dose response curve is flatter than that of H 56/28, Ph QA 33 and propranolol, a CNS-depressant effect of MJ 1999 must be taken into consideration. Also in favour of this view is the finding by LISH *et al.* (1965) that the compound caused a decrease of the spontaneous activity in mice at doses greater than 40 mg/kg given intraperitoneally. MENNEAR & RUDZIK (1965) also reported a complete protection against d-amphetamine toxicity in aggregated mice at a dose of 30 mg/kg MJ 1999 given intraperitoneally.

As to the relationship between the CNS-depressant properties

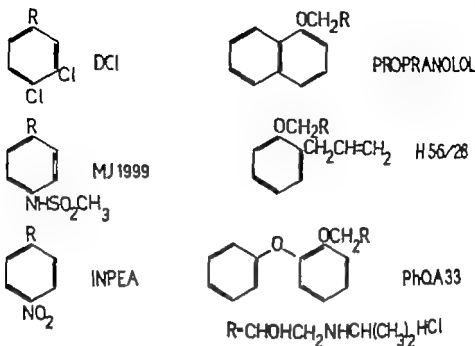


Fig. 1 The chemical structure of some β -adrenergic blocking compounds.

β -adrenergic blockade, the present evidence indicates that the two parameters are not correlated. MURMANN *et al* (1966) showed that the β -adrenergic blocking (—) isomer of INPEA was not CNS-depressant while the adrenergic inactive (+) isomer was excitatory. As INPEA is a considerably weaker β -adrenergic blocking agent than propranolol (HERMANSEN 1969), the optical isomers of the latter compound may be more suitable for the study of a possible relationship between CNS depression and β -blockade. Table 2 shows that (+)- and (—)-propranolol are equiactive with regard to the barbiturate potentiating effect, although the β -blocking effect of the former compound is less than 1/ of the latter (BARRER & CULLUM 1968). Another observation which provides evidence against a relationship between the two features, is the different dose levels at which the effects are produced. The antagonistic action of Ph QA 33 and propranolol against isoprenaline induced tachycardia in mice is thus displayed at a dose level of 1–3 mg/kg given intraperitoneally i.e. doses which are about 10/ of the CNS depressing doses (HERMANSEN 1968).

The possible clinical relevance of the results may be supported by the fact that a rather high incidence of depressive symptoms has been encountered during a trial with propranolol on hypertensive patients (WAAL 1967). These observations have been questioned by FITZGERALD (1967).

who argues that the clinical experience from the world wide use of propranolol indicates an incidence of depression not higher than 0.1%. The CNS depressant properties of several β -adrenergic blocking agents as demonstrated by their barbiturate potentiating effect may therefore be regarded as marginal effects.

Summary

- 1 The β -adrenergic blocking compounds H 56/28 Ph QA 33 and propranolol have been demonstrated to potentiate barbiturate induced sleep in mice and found to be equally potent. MJ 1999 was considerably weaker while INPEA had no effect. This indicates that a potential CNS depressant action is not limited to a special chemical configuration of β -receptor blocking compounds.
- 2 The two optical isomers of propranolol showed a similar barbiturate potentiating effect despite the fact that the (+) isomer is devoid of β -blocking effect. This suggests that CNS depression and β -blockade are unrelated phenomena.
- 3 The significance of the CNS-depressant effect is discussed particularly in relation to the β -blocking effect and toxicity of the compounds.

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Tachyphylaxis and Toxicity of 5HT in Anaesthetized Rats

By

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(Received June 9 1969)

During anaesthesia the intravenous toxicity of 5 HT (5-hydroxytryptamin, serotonin) is greatly increased (CORRELL *et al* 1952). When analyzing the intravenous toxicity of 5 HT more closely in anaesthetized rats (TAMMISTO 1965) it was found that during moderate surgical anaesthesia (e.g. pentobarbital 40 mg/kg (mebumalum NFN) intraperitoneally), the toxicity increased 600 times (i.e. LD₅₀ from 60 to 0.1 mg/kg) and the slope of the dose-mortality curve was relatively steep. The slope of the dose-mortality curve, however, always decreased when the curve shifted to the right while the toxicity of 5 HT was partially antagonized by various procedures. An almost bimodal dose mortality curve was sometimes even found. 5 HT has been shown to desensitize the tryptamine receptors in different tissues to the actions of 5 HT (GADDUM 1953 GINZEL & KOTTEGODA 1954 KOELLA & CZICMAN 1963 & 1966 TAMMISTO 1965). Since a decrease in the slope of the dose-mortality curve might be due to desensitization an attempt was made to find out if 5 HT itself or tryptamine could antagonize the lethal actions of 5 HT. Attention was also paid to the toxicity modifying actions of some drugs which affect the turnover of 5 HT and to the toxic effects of tryptamine. Some of these results have been presented at the second annual meeting of the Finnish Medical Societies held in Helsinki on March 28-29 1969.

Methods

Adult white male Sprague-Dawley rats weighing 100-150 g were used. The animals were not fasted, and received standard laboratory diet and water *ad libitum*. When determining the toxicity 5 HT or tryptamine was injected into the tail vein 20 min. after the intraperitoneal administration of 40 mg/kg of pentobarbital. The amount of the solution 1

was, whenever possible, 0.2 ml/100 g. The injection was given over a period of 3-5 sec. The environmental temperature was 22-23°. Groups of 5 or 10 animals are used. Deaths occurring during anaesthesia were recorded. The LD₅₀ values and their standard errors (S.E.) were estimated by the graphical method of MILLER & TADNER (1944).

The desensitization doses (in 0.2 ml/100 g) of 5 HT or tryptamine were injected subcutaneously at varying intervals before the intravenous dose of 5 HT.

The following drugs were used: p-chlorophenylalanine (p-CPA) (Sigma, Chemical Company), DL-5-hydroxytryptophan (5-HTP) (Fluka AG), imipramine (Janssen & Co., J. R. Geigy AG), neostigmine methylsulphate (prostigmin®; F. Hoffmann-La Roche & Co. AG), nialamide (niamid®; Chas. Pfizer & Co., Inc.), nicotine (distilled from the concentrated solution of nicotine sulphate and dissolved in saline and neutralized with hydrochloric acid to pH 7), phentolamine hydrogen sulphate (Leo AB), physostigmine salicylate (commercial sample), reserpine (reserpin®; Lääke Oy), and tetrabenazine hydrochloride (F. Hoffmann-La Roche & Co. AG). 5-HT was given as the creatinine sulphate (acetonide-creatinesulphat monohydrat, Fluka AG) and tryptamine as the hydrochloride (tryptaminhydrochlorid, Fluka AG). The doses refer to the base.

Results

Table 1 shows the death rates caused by 1 mg/kg of intravenously administered 5-HT during pentobarbital anaesthesia after different subcutaneously administered desensitizing doses of 5-HT or tryptamine. On the basis of earlier studies (TAMMISTO 1965) 1 mg/kg was chosen as the intravenous test dose since the LD₅₀ of 5-HT during pentobarbital anaesthesia (40 mg/kg) was found to be 0.1 mg/kg while 1 mg/kg of 5-HT caused a 100% mortality which could also be ascertained in the present experiments. It can be seen that 0.5 mg/kg of 5-HT has already a slight tachyphylactic action which lasts for about 60 min. The efficacy and duration of the tachyphylaxis increases with higher desensitizing doses and 10 mg/kg of 5-HT almost completely abolishes the mortality when the desensitization interval varies from 60 to 90 min. After an interval of 150 min. the tachyphylactic effect of 10 mg/kg of 5-HT is still quite clear while that of 1 mg/kg is no longer evident.

The LD₅₀ of 5-HT was estimated after a desensitizing dose of 10 mg/kg with a conditioning interval of 60 min. and was found to exceed 25 mg/kg. The manner of death of the desensitized rats resembled that occurring in the controls, i.e. apnoea, laboured breathing and asphyxia usually in the course of 15 min.

Higher desensitizing doses were not used, since 10 mg/kg markedly deepened the subsequent pentobarbital anaesthesia. Particularly in groups in which the desensitizing dose was given 10 min. before pentobarbital (i.e. conditioning interval 30 min.) many rats were severely cyanosed and showed gasping respiratory movements during the intravenous administration of 5-HT.

Table 1

Death rate caused by 1 mg/kg of intravenous 5 HT in anaesthetized rats (pentobarbital 40 mg/kg intraperitoneally 20 min. earlier) after desensitization with 5 HT or tryptamine.

Desensitization Dose (mg/kg, s.c.)	Desensitization interval (min.)			
	30	60	90	150
Saline	10/10	5/5	5/5	-
5 HT				
0.2	-	9/10	-	-
0.5	4/10	5/10	8/10	-
1.0	7/10	5/10	4/10	5/5
10.0	2/10	1/10	0/10	2/10
Tryptamine				
10.0	-	8/10	-	-
25.0	-	5/10	-	-
50.0	-	1/10	-	-

Tryptamine also caused desensitization but the smallest effective doses were 25 to 50 times higher than the corresponding desensitizing doses of 5 HT (table 1). The doses required for an almost complete inhibition of the lethal actions of 1 mg/kg of 5 HT were about 5 times higher than the corresponding doses of 5 HT.

In the conscious controls the intravenous toxicity of tryptamine was about the same as that of 5 HT (LD₅₀ between 75 and 100 mg/kg as against 60 ± 8.5 mg/kg respectively). In this case too the manner of death resembled that occurring after 5 HT i.e. convulsions lasting for 30-60 sec. followed by terminal apnoea of about 2 min. duration before cardiac arrest. The convulsions, however, were more marked and showed the typical hind leg spread with arched back and fore paw clonus. During pentobarbital the toxicity of tryptamine increased only about 10-fold but the manner of death was similar to that after 5 HT. During pentobarbital therefore, 5 HT was at least 50-times as toxic as tryptamine (LD₅₀ 0.1 ± 0.01 mg/kg as against 8.5 ± 2.1 mg/kg).

Nicotine, neostigmine or physostigmine did not prevent the toxicity of 5 HT during pentobarbital anaesthesia (40 mg/kg). When nicotine (2 mg/kg, subcutaneously) neostigmine or physostigmine both (0.1 mg/kg, subcutaneously) were given 45 min. earlier 1 mg/kg of intravenous 5 HT caused a 100% mortality in the anaesthetized animals.

Table 2 shows the death rates caused by 1 mg/kg of 5 HT intravenously administered into anaesthetized rats following the administration of some drugs which affect the turnover of 5 HT. 5-HTP pretreatment gave

1965) the central actions of 5 HT have been assumed to be the cause of the respiratory failure. This assumption is also supported by the present finding that tetrabenazine and reserpine gave a similar protection. On the other hand effective desensitization to the effects of 5 HT has only been demonstrated with certainty in peripheral actions.

VOGT (1968) has recently discussed the cerebral tryptamine receptors and the possible explanations for the different effects produced by 5 HT tryptamine or its derivatives. The existence of more than one type of tryptamine receptor with different affinities for each compound and the existence of different sites of action would both offer a plausible explanation for the finding that 5 HT is 50 times more toxic than tryptamine during anaesthesia but that both are approximately equally toxic in intact rats. It could be speculated that the affinity of the "stimulatory" receptors is similar both for 5 HT and tryptamine, whereas the "inhibitory" receptors have a greater affinity for 5 HT than for tryptamine. Small doses of 5 HT (up to 1 mg/kg) might mainly affect "inhibitory" receptors which potentiate the effects of anaesthetics and thus cause death during anaesthesia. Increasing the dose desensitizes the "inhibitory" receptors hence the stimulatory responses becomes evident and the unanaesthetized animals die in convulsions. The "inhibitory" and "stimulatory" responses need not be elicited by different receptors but by the same receptors located at different sites. Thus KOELLA & CZICHAN (1966) have suggested that the EEG synchronizing effect of 5 HT might originate in the area postrema and the desynchronizing effect somewhere more rostrally in the structures outside the blood brain barrier.

According to UDENFRIEND *et al* (1957) 150 mg/kg of 5 HTP increased the brain 5 HT level of rat by about 6 times. Twice this dose of 5 HTP was required to produce a similar desensitization as that produced by 1 mg/kg of 5 HT. The weak effect of 5 HTP might be explained by the finding of BOGDANSKI *et al* (1958) that much of the 5 HT formed in rat uterus is present in a pharmacologically inactive state or that its formation is mainly restricted to the vascular endothelium in the central nervous system (BERTLER *et al* 1963).

Though the reserpine syndrome seems to be mainly due to loss of dopamine (ANDÉN 1968) the sedative action may be due to the release of endogenous 5 HT (BRODIE & SHORE 1957) and mediated through a constant occupancy of 5 HT receptors (BRODIE & REID 1968). Accordingly the reduction in the toxicity of 5 HT during anaesthesia, observed after pretreatment with reserpine or tetrabenazine could be due to the desensitization of 5 HT receptors. The relatively weak effect might be due to the rapid inactivation of the released 5 HT before it reaches the receptors.

The results obtained with reserpine after 5 HTP administration are inconclusive. According to the "desensitization theory" a better protection against 5 HT toxicity would have been anticipated but on the other hand 5 HTP is known to protect the 5 HT stores against reserpine (CARLSSON 1967).

Loss of 5 HT hardly accounts for the reduced toxicity of 5 HT since pretreatment with p-CPA, which has been shown to reduce the brain 5 HT content to one tenth of the control level (KOE & WEISSMANN 1966) did not reduce the toxicity of 5 HT. Many other actions, however, may be responsible for the reduced toxicity of 5 HT after reserpine since blockade of the incorporation into granules also affects other monoamines.

Elevation of the 5 HT content by MAO inhibitors or blockade of the 5 HT uptake by imipramine (CARLSSON *et al* 1968) did not reduce the toxicity of 5 HT during anaesthesia but on the contrary seemed to increase it. Some other responses to 5 HT are also potentiated by MAO inhibitors (BOROWITZ & NORTH 1959) or by imipramine (GYERMEX & POSSEMATO 1960). It is possible that the inhibited inactivation of 5 HT by blockade of the enzyme or membrane pump increases the effect of injected 5 HT to such an extent, that the slight protection possibly caused by desensitization remains insignificant. However since MAO inhibitors and imipramine reverse the effects of reserpinization, they may possibly somehow change the reactivity of 5 HT receptors. This change might offer a more probable explanation for the lack of desensitization by MAO inhibitors or by imipramine.

Summary

The protective action of administered 5 HT or tryptamine subcutaneously against the intravenous toxicity of 5 HT during pentobarbital anaesthesia was studied in rats. As little as 0.5 mg/kg of 5 HT had a slight tachyphylactic action which lasted for about 1 hr. Higher doses of 5 HT increased the efficacy and duration of the tachyphylaxis. Pretreatment with 10 mg/kg of 5 HT elevated the intravenous LD₅₀ of 5 HT during pentobarbital anaesthesia by more than 250 times and the desensitization lasted for over 2½ hrs. Tryptamine also caused desensitization but it was about 20 times less effective than 5 HT. Tryptamine was about 50 times less toxic than 5 HT during pentobarbital anaesthesia though both were equally toxic in unanaesthetized rats. Pretreatment with nicotine or physostigmine had no effect. Reserpine, tetrabenazine or 5 HTP pretreatment antagonized the toxicity of 5 HT whereas p-CPA, nialamide, phenelzine or imipramine gave no protection.

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Magnesium and Calcium Metabolism during Prolonged Furosemide (Lasix ®) Administration to Normal Rats

By

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A marked increase in urinary magnesium and calcium excretion has been reported during intravenous furosemide (Lasix ®) infusions into anaesthetized dogs (DUARTE 1968). To elucidate whether prolonged furosemide administration might induce magnesium depletion or whether renal and/or intestinal regulatory mechanisms counteract the initial urinary losses, a magnesium balance study was carried out during chronic furosemide treatment of normal rats. The urinary calcium excretion was studied simultaneously.

Materials and Methods

White male Wistar rats from Møllegaard Hansen, Breeding Centre (mean weight 230 ± 8.0 g) were kept in individual metabolic cages at 22°. They were fed Altromin rat powder, an artificial diet and given redistilled water ad libitum. The degree of food loss from the food beaker to the cage was minimal, as the opening to the food beaker was so small that it only allowed the passage of the head of the rat. Food contamination of the urine was further prevented by a grid placed below the faeces grid. The inside of the metal cages was covered with polyethylene funnels. The grids and the funnels were changed every 24 hours. Urine was collected in glass tubes with graduated scale which allowed readings for 100 µl. After a training period of six days, an experimental period of 18 days was started. This was subdivided into a control period, a period of treatment and a period after cessation of treatment each of six days duration.

The food was dried at 120° and weighed. The mean magnesium and calcium concentrations of the food \pm S.D. of all different samples of the Altromin food batch, were 0.131 ± 0.03 and 0.663 ± 0.043 meq/g dry food, respectively. The magnesium concentration in the present batch was 6.5 per cent higher and the calcium concentration 16.0 per cent higher than the average value given by the manufacturer. According to the manufacturer the

sodium content of the food was 52 µeq./g food. Another 75 µeq sodium (as sodium chloride) was added per g food.

Furosemide was given orally in the same dose as used in a previous study on the natriotic effect (5 mg/g food) (STEVEN & SKADHAUGE 1969). Thorough mixing with water is ensured. The resulting food suspension consisting of 20 g of food and 30 ml of water remained in a paste-like consistency for 24 hours. As the water content of the food was found to be about 10 per cent of the total weight, the rats were given approximately 12 g of dry food daily.

Analyses

Magnesium and calcium were determined by atomic absorption spectrophotometry (Perkin Elmer Atomic Absorption Spectrophotometer 290 acetylene-air flame, three-dot Boling burner). One per cent Lanthanum oxide was used for the preparation of standards and dilutions in order to obviate phosphate interference on the calcium readings. The coefficient of variation for magnesium and calcium determinations of serum pool analysed once a week before and during the experimental period was 3.6 per cent ($n = 22$) and 1.4 per cent ($n = 9$), respectively. All determinations including those of the faeces were made in duplicate.

Urine analysis

The urine was acidified immediately after the collection of samples by addition of concentrated hydrochloric acid (50 µl per ml urine). The determination of calcium by atomic absorption spectrophotometry is known to be dependent on the pH, i.e. low pH values cause a decrease in the readings, while the effect on the magnesium determination is slight (MOWBR & SEXTON 1967). This has been confirmed by the authors. The pH of 10 urine dilutions and 12 different faeces samples, ashed and dissolved as described, was compared with the pH of 5 different standards and another 5 different standards, ashed and dissolved as described for faeces. No difference was noted (pH 0.80 in all the cases). The replicates coefficient of variation of the urine analysis was 2.4 per cent ($n = 40$) for magnesium and 0.7 per cent ($n = 40$) for calcium. The mean recovery of magnesium added to urine was $100.1 \pm \text{S.D. } 2.6$ per cent ($n = 44$) and that of calcium $97.9 \pm \text{S.D. } 2.3$ per cent ($n = 44$).

Analysis of the faeces

The faeces were collected daily and 3 day samples from each animal were analysed for magnesium and calcium. Drying was performed at 120°. After drying for 24 hrs, the faeces were ground to fine powder mixed, and dried for another 3–5 days until the weight was constant after cooling in a desiccator. Ashing was performed as follows: 2 g of the whole amount of the dry faeces powder sample was suspended in 30 ml of a solution consisting of equal parts of 70 per cent perchloric acid and 65 per cent nitric acid. After stirring for 20 minutes on a magnetic stirring table, 500 µl of the suspension were transferred in pyrex glass tubes and wet ashing was performed in an induction block at 180° after slow heating for 3 hours (FLATTINGBERG *et al.* 1966). After ashing for 34 hrs the ash was dissolved in 100 µl conc. hydrochloric acid and diluted. The coefficient of variation for the magnesium analysis in the faeces was 1.3 per cent. The mean recovery of magnesium added to the faeces samples before ashing was $101.0 \pm \text{S.D. } 1.9$ per cent ($n = 8$).

Blood samples

Blood was drawn by cardiac puncture from group I 20 male rats (mean weight 230 g) after 20 hours of furosemide treatment with the same dose of furosemide as in the urinary excretion experiments, and from a control group consisting of another 20 rats of the same weight treated similarly except that no furosemide was administered. The blood was collected in glass tubes, without anticoagulants. Centrifugation and separation of the serum was carried out within 30 minutes and the serum magnesium and calcium determined.

Only distilled water twice redistilled in a Horrocks all quartz bidistillation apparatus was used for dilutions, for mixing the food, and for the preparation of reagents.

Results

Preliminary experiments with a less refined experimental technique than described above and with another batch of "Altromm" food powder confirmed the findings of DUARTE (1968) with regard to the effect of furosemide on urinary magnesium excretion. Thus, after oral furosemide administration to eleven normal 220 g male Wistar rats for 24 hours, the urinary magnesium excretion was $24.3 \pm S.D. 9.3 \mu\text{eq/g food}$ and that of the basal period (4 days) $6.0 \pm S.D. 2.8 \mu\text{eq/g food}$.

The daily urinary excretion of magnesium and calcium found with the experimental technique described, was markedly augmented during the whole period of treatment whether expressed as total urinary magnesium and calcium excretion or as urinary magnesium and calcium excretion per g food (fig. 1) ($P < 0.01$ and $P < 0.001$ respectively for each day of the treatment period calculated by Student's *t* test). The maximum urinary excretion of both metals was found on the second day in all the animals. The magnesuric and calciuric effect diminished gradually during continued furosemide treatment. The urinary magnesium and calcium losses were not compensated for by a reduced urinary excretion in the post treatment period. The urinary flow and the urine calcium excretion were also increased on the first post treatment day indicating that the duration of the effect of furosemide was about 24 hours (table 1).

There was a significant correlation between the urinary magnesium and calcium excretion during the period of treatment (fig. 2).

The urinary excretion of calcium, expressed as $\mu\text{eq Ca/24 hrs} \times \text{g food per ml urine}$ was either slightly augmented or unchanged during the period of treatment whereas that of magnesium was markedly decreased (table 1).

The weight losses during treatment are shown in fig. 1. The mean basal weight of the rats (233.9 g) was reached again on the second day of the post treatment period.

The average food intake during the basal period, the treatment per-

and the post treatment period was $16.5 \pm \text{S.D. } 11$ $11.7 \pm \text{S.D. } 4.2$, and 16.2 ± 1.9 g/24 hrs, respectively. The physical activity of the rats was apparently reduced during treatment.

The mean magnesium balances of the six periods of the experiment in $\mu\text{eq}/72$ hrs ($\pm \text{S.D.}$) (= magnesium intake - (urinary magnesium losses + faecal magnesium losses)) were as follows I 1075.6 (± 269.4) II 1414.8 (± 457.1) III 929.4 (± 479.1) IV 952.2 (± 565.9) V 1016.4 (± 437.1) and VI 874.4 (± 348.5). Thus, the magnesium balance was positive and practically unchanged during the whole experiment. The intestinal absorption of magnesium (magnesium intake - magnesium losses in the faeces) per g food was significantly increased during treatment (table 2, fig. 3). The increase of magnesium absorption equaled the increase of urinary magnesium excretion. The magnesium absorption in the post treatment period did not differ significantly from the basal magnesium absorption.

No significant changes in serum magnesium or serum calcium were observed after furosemide treatment for 20 hours (table 3).

Intestinal Absorption
 $\mu\text{eq Mg}/72\text{hrs} \times \text{g food} \pm \text{s.e.}$

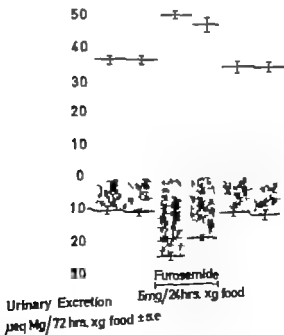


Fig. 3 The intestinal absorption of magnesium during furosemide administration as related to the urinary magnesium excretion. Periods of 72 hours.

Table 2
Intestinal absorption and urinary excretion of magnesium six periods of 72 hours. (Average of 10 rats).

	Absorption (Intake - fecal excretion) $\frac{mg}{72 \text{ hrs}} \pm S.D.$	P	Urinary excretion $\frac{mg}{72 \text{ hrs}} \pm S.D.$	P	Increase of absorption $\frac{mg}{72 \text{ hrs}} \pm S.D.$	Increase of urinary excretion $\frac{mg}{72 \text{ hrs}} \pm S.D.$	P
Basal period	36.0 ± 7.3		11.0 ± 3.4				
Medication period	49.5 ± 4.9	< 0.01	25.7 ± 4.6	< 0.001	$+13.5 \pm 3.8$	$+14.7 \pm 4.6$	> 0.1
	45.5 ± 9.5	< 0.01	20.4 ± 5.4	< 0.001	$+10.6 \pm 9.6$	$+9.4 \pm 5.4$	> 0.1
Post medication period	33.2 ± 7.8	> 0.1	12.4 ± 3.7	> 0.1			
	33.0 ± 7.0	> 0.1	13.9 ± 3.9	> 0.05			

Student's t-test for significance of difference between mean values and mean basal value.

Student's t-test for significance of difference between increase of absorption and increase of urinary excretion.

sodium content of the food was 52 $\mu\text{eq./g}$ food. Another 75 μeq sodium (as sodium chloride) was added per g food.

Furosemide was given orally in the same dose as used in a previous study on the natriotic effect (5 mg/g food) (STEVEN & SKADHAUGE 1969). Thorough mixing with water was ensured. The resulting food suspension consisting of 20 g of food and 30 ml of water remained in a pastelike consistency for 24 hours. As the water content of the food was found to be about 10 per cent of the total weight, the rats were given approximately 18 g of dry food daily.

Analyses

Magnesium and calcium were determined by atomic absorption spectrophotometry (Perkin Elmer Atomic Absorption Spectrophotometer 290 acetylene-air flame, three-slot Boling burner). One per cent Lanthanum oxide was used for the preparation of standards and dilutions in order to obviate phosphate interference on the calcium readings. The coefficient of variation for magnesium and calcium determinations of a serum pool analyzed once a week before and during the experimental period was 1.6 per cent ($n = 22$) and 1.4 per cent ($n = 9$), respectively. All determinations including those of the faeces were made in duplicate.

Urine analysis

The urine was acidified immediately after the collection of samples by addition of concentrated hydrochloric acid (30 μl per ml urine). The determination of calcium by atomic absorption spectrophotometry is known to be dependent on the pH, i.e. low pH values cause a decrease in the readings, while the effect on the magnesium determination is slight (MOORE & SIGGS 1967). This has been confirmed by the authors. The pH of 10 urine dilutions and 12 different faeces samples, ashed and dissolved as described, was compared with the pH of 5 different standards and another 5 different standards, ashed and dissolved as described for faeces. No difference was noted (pH 0.80 in all the cases). The replicate coefficient of variation of the urine analysis was 2.4 per cent ($n = 40$) for magnesium and 0.7 per cent ($n = 40$) for calcium. The mean recovery of magnesium added to urine was $100.1 \pm \text{S.D. } 2.6$ per cent ($n = 44$) and that of calcium $97.9 \pm \text{S.D. } 2.3$ per cent ($n = 46$).

Analysis of the faeces

The faeces were collected daily and 3 day samples from each animal were analyzed for magnesium and calcium. Drying was performed at 120°. After drying for 24 hrs, the faeces were ground to fine powder mixed, and dried for another 3-5 days until the weight was constant after cooling in desiccator. Ashing was performed as follows: 2 g of the whole amount of the dry faeces powder sample was suspended in 30 ml of a solution consisting of equal parts of 70 per cent perchloric acid and 65 per cent nitric acid. After stirring for 20 minutes on a magnetic stirring table, 300 μl of the suspension were transferred to pyrex glass tubes and wet ashing was performed in an aluminium block at 210° after slow heating for 3 hours (HARTNUNG *et al.* 1968). After ashing for 36 hrs the ash was dissolved in 100 μl conc. hydrochloric acid and diluted. The coefficient of variation for the magnesium analysis in the faeces was 1.3 per cent. The mean recovery of magnesium added to the faeces samples before ashing was $101.0 \pm \text{S.D. } 1.9$ per cent ($n = 8$).

Blood samples

Blood was drawn by cardiac puncture from a group of 20 male rats (mean weight 230 g) after 20 hours of furosemide treatment with the same dose of furosemide as in the urinary excretion experiments, and from a control group consisting of another 20 rats of the same weight treated similarly except that no furosemide was administered. The blood was collected in glass tubes, without anticoagulants. Centrifugation and separation of the serum was carried out within 30 minutes and the serum magnesium and calcium determined.

Only distilled water twice redistilled in a Heraeus[®] all quartz bidistillation apparatus was used for dilutions, for making the food and for the preparation of reagents.

Results

Preliminary experiments with a less refined experimental technique than described above and with another batch of "Altromin" food powder confirmed the findings of DUARTE (1968) with regard to the effect of furosemide on urinary magnesium excretion. Thus after oral furosemide administration to eleven normal 220 g male Wistar rats for 24 hours, the urinary magnesium excretion was $24.3 \pm \text{S.D. } 9.3 \mu\text{eq/g food}$ and that of the basal period (4 days) $6.0 \pm \text{S.D. } 2.8 \mu\text{eq/g food}$.

The daily urinary excretion of magnesium and calcium found with the experimental technique described, was markedly augmented during the whole period of treatment whether expressed as total urinary magnesium and calcium excretion or as urinary magnesium and calcium excretion per g food (fig. 1) ($P < 0.01$ and $P < 0.001$ respectively for each day of the treatment period calculated by Student's *t*-test). The maximum urinary excretion of both metals was found on the second day in all the animals. The magnesuric and calciuric effect diminished gradually during continued furosemide treatment. The urinary magnesium and calcium losses were not compensated for by a reduced urinary excretion in the post treatment period. The urinary flow and the urine calcium excretion were also increased on the first post treatment day indicating that the duration of the effect of furosemide was about 24 hours (table 1).

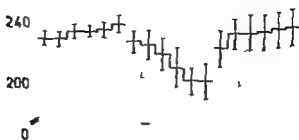
There was a significant correlation between the urinary magnesium and calcium excretion during the period of treatment (fig. 2).

The urinary excretion of calcium, expressed as $\mu\text{eq Ca}/24 \text{ hrs} \times \text{g food}$ per ml urine was either slightly augmented or unchanged during the period of treatment whereas that of magnesium was markedly decreased (table 1).

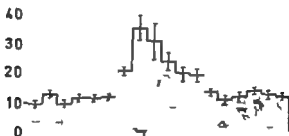
The weight losses during treatment are shown in fig. 1. The mean basal weight of the rats (233.9 g) was reached again on the second day of the post treatment period.

The average food intake during the basal period, the treatment period,

Mean rat weight \pm s.d.(g)



μ eq Mg/24hrs x g food \pm s.e.



μ eq Ca/24hrs x g food \pm s.e.

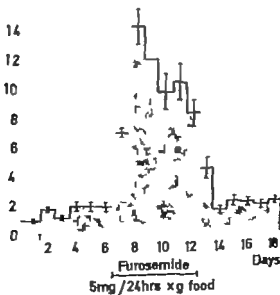


Fig 1 The urinary excretion of magnesium and calcium before, during, and after furosemide administration.

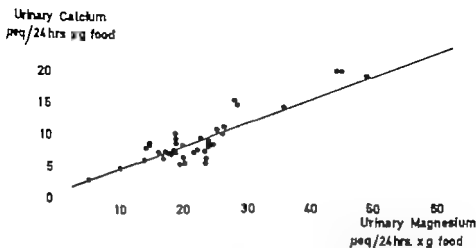


Table 1

Daily urinary magnesium and calcium excretion per ml urine.

Treatment from the 7th-12th day

Urine flow ml/24 hrs \pm S.D.	meq Mg/24 hrs x g food per ml urine (average of 10 rats)	meq Ca/24 hrs x g food per ml urine (average of 10 rats)
5.9 \pm 1.8	1.58	0.17
8.8 \pm 2.3	1.47	0.21
7.0 \pm 1.7	1.33	0.17
9.5 \pm 2.8	1.20	0.12
8.1 \pm 1.7	1.41	0.25
9.2 \pm 1.5	1.30	0.22
35.3 \pm 5.2	0.99	0.20
47.8 \pm 11.5	0.74	0.30
41.5 \pm 12.1	0.76	0.29
40.9 \pm 6.8	0.39	0.24
51.1 \pm 16.8	0.40	0.21
39.1 \pm 16.8	0.50	0.22
25.7 \pm 8.1	0.54	0.18
7.3 \pm 1.0	1.52	0.23
10.3 \pm 1.7	1.16	0.22
13.3 \pm 1.8	1.08	0.17
10.6 \pm 2.3	1.25	0.19
9.6 \pm 1.4	1.29	0.24

and the post treatment period was $16.5 \pm S.D. 11$ $11.7 \pm S.D. 4.2$, and 16.2 ± 1.9 g/24 hrs, respectively. The physical activity of the rats was apparently reduced during treatment.

The mean magnesium balances of the six periods of the experiment in $\mu\text{eq}/72 \text{ hrs}$ ($\pm S.D.$) ($=$ magnesium intake $-$ (urinary magnesium losses $+$ faecal magnesium losses)) were as follows: I 1075.6 (± 269.4) II 1414.8 (± 457.1) III 929.4 (± 479.1) IV 952.2 (± 565.9) V 1016.4 (± 437.1) and VI 874.4 (± 348.5). Thus, the magnesium balance was positive and practically unchanged during the whole experiment. The intestinal absorption of magnesium (magnesium intake $-$ magnesium losses in the faeces) per g food was significantly increased during treatment (table 2, fig. 3). The increase of magnesium absorption equaled the increase of urinary magnesium excretion. The magnesium absorption in the post treatment period did not differ significantly from the basal magnesium absorption.

No significant changes in serum magnesium or serum calcium were observed after furosemide treatment for 20 hours (table 3).

Intestinal Absorption
 $\mu\text{eq Mg}/72 \text{ hrs} \times \text{g food} \pm \text{s.e.}$

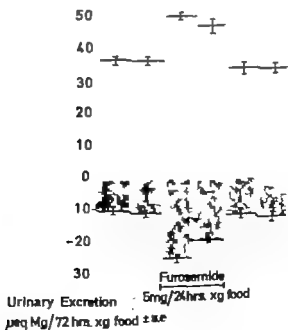


Fig. 3 The intestinal absorption of magnesium during furosemide administration related to the urinary magnesium excretion. Periods of 72 hours.

Table 2

Intestinal absorption and urinary excretion of magnesium. Six periods of 72 hours. (Average of 10 rats).

	Absorption (faecal excretion)	Urinary excretion	Increase of absorption	Increase of urinary excretion	P
	$\frac{mg}{72 \text{ hrs}} \frac{g \text{ food}}{g \text{ food}} \pm S.D.$	$\frac{mg}{72 \text{ hrs}} \frac{g \text{ food}}{g \text{ food}} \pm S.D.$	$\frac{mg}{72 \text{ hrs}} \frac{g \text{ food}}{g \text{ food}} \pm S.D.$	$\frac{mg}{72 \text{ hrs}} \frac{g \text{ food}}{g \text{ food}} \pm S.D.$	
Basal period	I 36.0 \pm 7.3	11.0 \pm 3.4			
Medicatio period	III 49.5 \pm 4.9	25.7 \pm 4.6	+13.5 \pm 5.8	+14.7 \pm 4.6	< 0.001
	IV 46.6 \pm 9.6	20.4 \pm 5.4	+10.6 \pm 9.6	+9.4 \pm 5.4	< 0.001
Post medication period	V 33.2 \pm 7.8	12.4 \pm 3.7			> 0.1
	VI 33.0 \pm 7.0	13.9 \pm 3.9			> 0.05

Student's t-test for significance of difference between mean value and mean basal value.

Student's t-test for significance of difference between increase of absorption and increase of urinary excretion.

and the post treatment period was $16.5 \pm \text{S.D. } 1.1$ and $17.4 \pm \text{S.D. } 4.2$, and 16.2 ± 1.9 g/24 hrs, respectively. The physical activity of the rats was apparently reduced during treatment.

The mean magnesium balances of the six periods of the experiment in $\mu\text{eq}/72 \text{ hrs}$ ($\pm \text{S.D.}$) (= magnesium intake - (urinary magnesium losses + faecal magnesium losses)) were as follows: I 1075.6 (± 269.4) II 1414.8 (± 457.1) III 929.4 (± 479.1) IV 952.2 (± 565.9) V 1016.4 (± 437.1), and VI 874.4 (± 348.5). Thus, the magnesium balance was positive and practically unchanged during the whole experiment. The intestinal absorption of magnesium (magnesium intake - magnesium losses in the faeces) per g food was significantly increased during treatment (table 2, fig. 3). The increase of magnesium absorption equaled the increase of urinary magnesium excretion. The magnesium absorption in the post treatment period did not differ significantly from the basal magnesium absorption.

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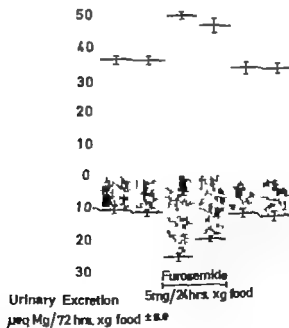


Fig. 3 The intestinal absorption of magnesium during furosemide administration as related to the urinary magnesium excretion. Periods of 72 hours.

Table 2

Intestinal absorption and urinary excretion of magnesium. Six periods of 72 hours. (Average of 10 rats).

	Absorption (Intake fecal excretion)	Urinary excretion	Increase of absorption	Increase of urinary excretion
	$\frac{\text{mg.}}{72 \text{ hrs.}} \pm \text{S.D.}$	$\frac{\text{mg.}}{72 \text{ hrs.}} \pm \text{S.D.}$	$\frac{\text{mg.}}{72 \text{ hrs.}} \pm \text{S.D.}$	$\frac{\text{mg.}}{72 \text{ hrs.}} \pm \text{S.D.}$
I and II	36.0 \pm 7.3	11.0 \pm 3.4		
Medication period	49.5 \pm 4.9	25.7 \pm 4.6	$+13.5 \pm 3.8$	$+14.7 \pm 4.0$
	46.6 \pm 9.6	20.4 \pm 5.4		
Post medication period	33.2 \pm 7.8	12.4 \pm 3.7	-10.6 ± 9.6	-14.4 ± 4.4
	33.0 \pm 7.0	13.9 \pm 3.9		
			< 0.001	< 0.001
			< 0.001	> 0.1
			> 0.1	~ 0.0

Student's *t*-test for significance of differences between means. Post and control for 1 h.
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Table 3

Serum concentrations of magnesium and calcium before and after treatment with furosemide for 20 hrs.

	Serum magnesium \pm S.D. meq./l	Serum calcium \pm S.D. meq./l
Control group (20 rats)	1.68 \pm 0.13	4.94 \pm 0.17
1st day of furosemide administration (20 rats)	1.75 \pm 0.10	4.95 \pm 0.14

Difference not significant by Student's *t*-test. ($P > 0.05$)

Discussion

The urinary excretion pattern of magnesium and calcium during chronic furosemide treatment differs markedly from that of sodium. An increased sodium excretion was seen only on the first day of treatment (STEVEN & SKADHAUGE 1968) whereas in the present work a significant increase in the magnesium and calcium excretion was seen on each day of the treatment period, the maximum increase being found on the second and third day. Hence a compensatory effect of salt and water retaining mechanisms, active during prolonged furosemide treatment, on the magnesium and calcium excretion, cannot *a priori* be ruled out. An increased rate of aldosterone secretion might be an important factor. It is well known that aldosterone enhances the urinary excretion of magnesium (WALSER 1967). Evidence that a secondary hyperaldosteronism is present during furosemide treatment has been given by LARAGH *et al* (1966). Likewise, in spite of an increased urine flow an increased vasopressin secretion rate brought about by a decrease in blood volume during treatment might be partly responsible for the increase in magnesium and calcium excretion. It is well known that vasopressin enhances the urinary excretion of magnesium (NIELSEN 1964) and calcium (THORN 1961) possibly by inhibiting tubular reabsorption. It has previously been shown that serum osmolality is unchanged during chronic furosemide treatment (STEVEN & SKADHAUGE 1969).

The main tubular process involved in the renal handling of magnesium is reabsorption. Tubular secretion probably does not take place in mammals (WALSER 1967). Therefore, an increased urinary magnesium excretion must be due either to an increased filtration of magnesium or to a decreased tubular reabsorption of magnesium.

As pointed out by WALSER (1966 & 1967) a main determinant in the rate of renal tubular magnesium reabsorption is the rate at which reab-

sorption of water occurs in those portions of the nephron proximal to or continuous with the sites at which magnesium is reabsorbed

Furosemide inhibits the sodium reabsorption, and thus the water reabsorption, primarily in the distal part of the nephron probably in the loop of Henle (BERLINER *et al* 1966)

Active transport of magnesium like that of calcium (LASSITER *et al* 1963) probably occurs in all parts of the nephron. Stop flow studies have revealed similar reabsorption patterns for magnesium calcium and potassium, all three cations being reabsorbed with the greatest avidity in the proximal part of the distal nephron (SAMMY *et al* 1960 WESSON & LAULER 1959 MURDAUGH & ROBINSON 1960), near the site of maximal sodium reabsorption (WESSON & LAULER 1959)

Furosemide possibly has a direct inhibitory effect on the reabsorption of magnesium and calcium at this site of the tubule. No effect of furosemide applied intratubularly on tubular water permeability has been demonstrated (ULLRICH *et al* 1966)

In the acute experiments of DUARTE (1968) the relative and absolute increment of renal calcium clearance seen after furosemide administration was greater than that of magnesium clearance. The explanation of this difference remains obscure. It has previously been found by microperfusion studies (VOGEL & STOECKERT 1967) that the percentage decrease in the reabsorption of calcium induced by furosemide exceeds that of sodium. This was also found by the present authors. Furthermore, we also found that the percentage increase in magnesium excretion was greater than that of sodium (STEVEN & SKADHAUGE 1969)

It seems probable that the decrease in body weight found during treatment was mainly due to a decrease in body water as the mean basal weight was already attained on the second day of the post treatment period, i.e. immediately after the diuretic effect of furosemide, which lasted for 24 hours, had ceased. It has previously been shown that the sodium chloride retention on the first two post treatment days amounted to 15 per cent of the exchangeable body sodium (STEVEN & SKADHAUGE 1969)

In contrast to sodium (STEVEN & SKADHAUGE 1969) magnesium and calcium are not retained by renal mechanisms in the post treatment period. No total body magnesium depletion took place during prolonged furosemide treatment, although the extra urinary magnesium losses during this period amounted to 7 per cent of the total body magnesium (SPRAY & WIDDOWSON 1950)

No explanation is available for the observed increase in intestinal magnesium absorption. As pointed out by WALSER (1967) water removal concentrates luminal magnesium and thereby promotes its absorption. Thus, the percentage of ingested magnesium absorbed increases, if water

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No explanation is available for the observed increase in intestinal magnesium absorption. As pointed out by WALSER (1967) water removal concentrates luminal magnesium and thereby promotes its absorption. Thus, the percentage of ingested magnesium absorbed increases, if water

absorption is augmented. SMITH (1963) showed that a linear relationship exists between transit time to the distal ileum and magnesium absorption. Unfortunately in the present work the fresh weight and the water content of fresh faeces were not determined.

Apart from intestinal water absorption and dietary factors, parathyroid hormone may be a major factor in the regulation of the intestinal absorption of magnesium (CARE & KEYNES 1964). Evidence that intestinal absorption of magnesium is increased in hyperparathyroidism is given by HEATON & PYRAH (1963). It has been suggested by MACINTYRE *et al.* (1963) that magnesium plays a significant physiological role, similar to that of calcium, in the regulation of parathyroid hormone secretion. Further evidence has been given by CARE *et al.* (1966). The possibility that parathyroid hormone is released to a greater extent than normal during chronic furosemide treatment is not ruled out by the fact that the serum concentrations of both calcium and magnesium are unchanged after furosemide treatment for 20 hours. A fall in serum magnesium and calcium might, during the diuretic therapy, have been masked by haemoconcentration. Haemoconcentration with a resulting increase in the protein bound magnesium and calcium fractions would be expected to occur early in the treatment. A lowering of ultrafiltrable serum magnesium and calcium concentration has, in fact, been reported during intravenous infusion of furosemide (DUARTE 1968), and this might indicate that the parathyroid hormone secretion rate is increased in this situation.

Summary

The urinary magnesium and calcium excretion in normal rats was significantly increased on each of the six days of furosemide treatment. The most pronounced rise was seen on the second day. A significant correlation between the daily urinary magnesium and calcium excretion was observed. The concentration of magnesium in the urine decreased markedly in contrast to the calcium concentration. The renal magnesium losses were entirely compensated for by an increase in intestinal magnesium absorption during the treatment. No changes in serum magnesium or serum calcium concentration occurred after furosemide treatment for 20 hours.

Acknowledgements

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